

Antimicrobial peptides: Immunomodulatory and therapeutic potential for use in Atlantic salmon (*Salmo salar*)

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DECLARATION

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Mark Blumhardt

Abstract

The continued use of antibiotics will undoubtedly lead to increases in antibiotic resistant bacteria and decreased effectiveness of antibiotic therapeutics. Therefore, a need exists for new therapeutic agents which effectively treat bacterial outbreaks but limit the ability of microbes to become resistant over time.

Antimicrobial peptides are innate defense peptides produced by multicellular organisms to combat a wide variety of pathogens present in the environment. They are naturally produced by the host, exhibit remarkably diverse structures and bioactivity both in and amongst species, and function through a variety of bactericidal mechanisms which limit bacterial resistance. These characteristics make them a potential source for the development of new anti-infective agents. The purpose of this thesis was to assess the antimicrobial activity and stimulatory potential of natural and synthetically derived peptides for use in Atlantic salmon (*Salmo salar*).

In this research, four peptides (P9-4, P11-5, P11-6, and Protegrin-1) were shown to be effective at inhibiting salmon pathogens *Yersinia ruckeri* and *Aeromonas hydrophila*, with minimum inhibitory concentrations (MIC) of less than 20 μM in saline conditions. Additionally, all peptides were effective against *Escherichia coli* (MIC between 2.5 μM -40 μM). The antimicrobial activity and haemolytic ability of these peptides was greatly reduced in the presence of serum, with limited haemolysis observed in erythrocytes incubated with 640 μM of each peptide. Furthermore, P9-4 and P11-5 were shown to significantly increase transcription of the chemokine interleukin-8 (IL-8) in serum cultures of Atlantic salmon peripheral blood leukocytes (PBL) following six hour *in vitro* stimulation. Results from these experiments suggest that the potent antimicrobial and conversely haemolytic ability, of peptides commonly seen in conventional media becomes more modest when subjected to more realistic biologically relevant conditions. Additionally, two peptides were capable of influencing expression of the chemokine IL-8 which plays a role in chemotaxis of immune cells. This provides preliminary evidence for the use of these peptides as immunostimulants.

The ability of antimicrobial peptides to modulate the functions of immune cells in Atlantic salmon was explored. Functional assays were used to assess the direct stimulatory capacity of antimicrobial peptides on the induction of phagocytosis, cell proliferation, and respiratory burst which play an important role in the immune system of teleost fish. Synthetic antimicrobial peptides were selected as well as the two known Atlantic salmon cathelicidins (asCATH1 and asCATH2) and examined in these experiments. The synthetic peptides K6L2W3 and HHC-10 were shown to significantly improve phagocytic ability, phagocytic index, and respiratory burst in head kidney leukocytes (HKLs). Additionally, HHC-10 was shown to significantly improve cell proliferation. Of the natural antimicrobial peptides, asCATH2 significantly improved phagocytosis, cell proliferation and respiratory burst whereas asCATH1 did not. Previous work involving these cathelicidins suggested that functional difference exists between these peptides and that asCATH2 may play a multifaceted biological role during infections. This work provides supplemental evidence to support that claim.

The findings in this thesis show that some antimicrobial peptides possess potent antimicrobial abilities while others act to modulate cells to improve pathogen destruction and infection clearance. This is in agreement with previous research indicating that peptides exhibit a structure-activity relationship and diverse mechanisms of action. Moreover, this research further supports the potential of antimicrobial peptides as a natural blueprint for new drug development with applications in aquaculture and showcases the versatility of these peptides in teleost fish.

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List of Abbreviations

Antimicrobial peptides	AMPs
ANOVA	analysis of variance
asCATH	Atlantic salmon cathelicidin
CLR	C-type lectin receptors
COX-2	cyclooxygenase
CpG-ODN	CpG oligodeoxynucleotides
DAMP	damage associated molecular pattern
DMSO	dimethyl sulfoxide
DPC	dodecyl-phosphocholine
GALT	gut-associated lymphoid tissue
HDP	host defense peptides
HKL	head kidney leukocytes
HNP1	human α -defensins
IFN	interferons
IL-1 α	interleukin 1 α
IL-1 β	interleukin 1 β
IL-8	interleukin-8
ILT	interbranchial lymphoid tissue
iNOS	nitric oxide synthase
L-15	Leibovitz's L-15 medium
LL-37	human cathelicidin
LPS	lipopolysaccharide
MHB	Mueller-Hinton broth
MHC-II	major histocompatibility complex class II molecules
MIC	minimum inhibitory concentration
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitro blue tetrazolium
NLR	NOD-like receptors
NNV	nervous necrosis virus
NO	nitric oxide
P2	piscidin-2
PA	phagocytic activity
PAMP	pathogen-associated molecular pattern
PBL	peripheral blood leukocytes
PBS	phosphate buffered saline
PG-1	protegrin-1
PGRP	peptidoglycan recognition proteins
PI	phagocytic index
PMA	phorbol myristate acetate
Poly I:C	polyinosinic:polycytidylic acid
PRR	pattern recognition receptors

QSAR	quantitative structure-activity relationship
ROI	reactive oxygen intermediates
TH1-5	tilapia hepcidin 1-5
TH2-3	tilapia hepcidin 2-3
TLR	toll-like receptors
TNF- α	tumor necrosis factor α
zfBD2	zebrafish β -defensin 2

Chapter 1: General Introduction

The aquatic environment is inhabited by a wide variety of pathogenic microorganisms which interact with fish. To combat this, teleost fish have had to evolve an immune system capable of detecting a wide variety of microbes, protecting against disease, and assisting in the maintenance of homeostasis. The immune system of teleost fish has traditionally been divided into the innate and the adaptive immune system (Figure 1). While many stand-alone functions exist within each system, there is a growing body of evidence that suggests interplay between the two. In general, the innate response is activated before the adaptive response and helps to determine the nature of subsequent responses [1]. The innate immune system is comprised of physical barriers, cellular components, and humoral responses [1, 2]. These function in the trafficking of immune cells to sites of infection, recognition, destruction, and clearance of pathogens or dead cells in an immediate manner [3]. This system, which is evolutionarily conserved amongst multicellular organisms, is non-specific and a relatively temperature independent response in poikilotherms [2]. The adaptive response works in conjunction with the innate response and also is composed of humoral and cellular components. This response is highly specific and aids in improved recognition of the pathogen and is a more finely-tuned immunological response but is biologically costly, time-consuming, and temperature dependant [4]. Furthermore, adaptive immunity is closely associated with immunological memory and the production of antibodies to combat systemic, prolonged infections.

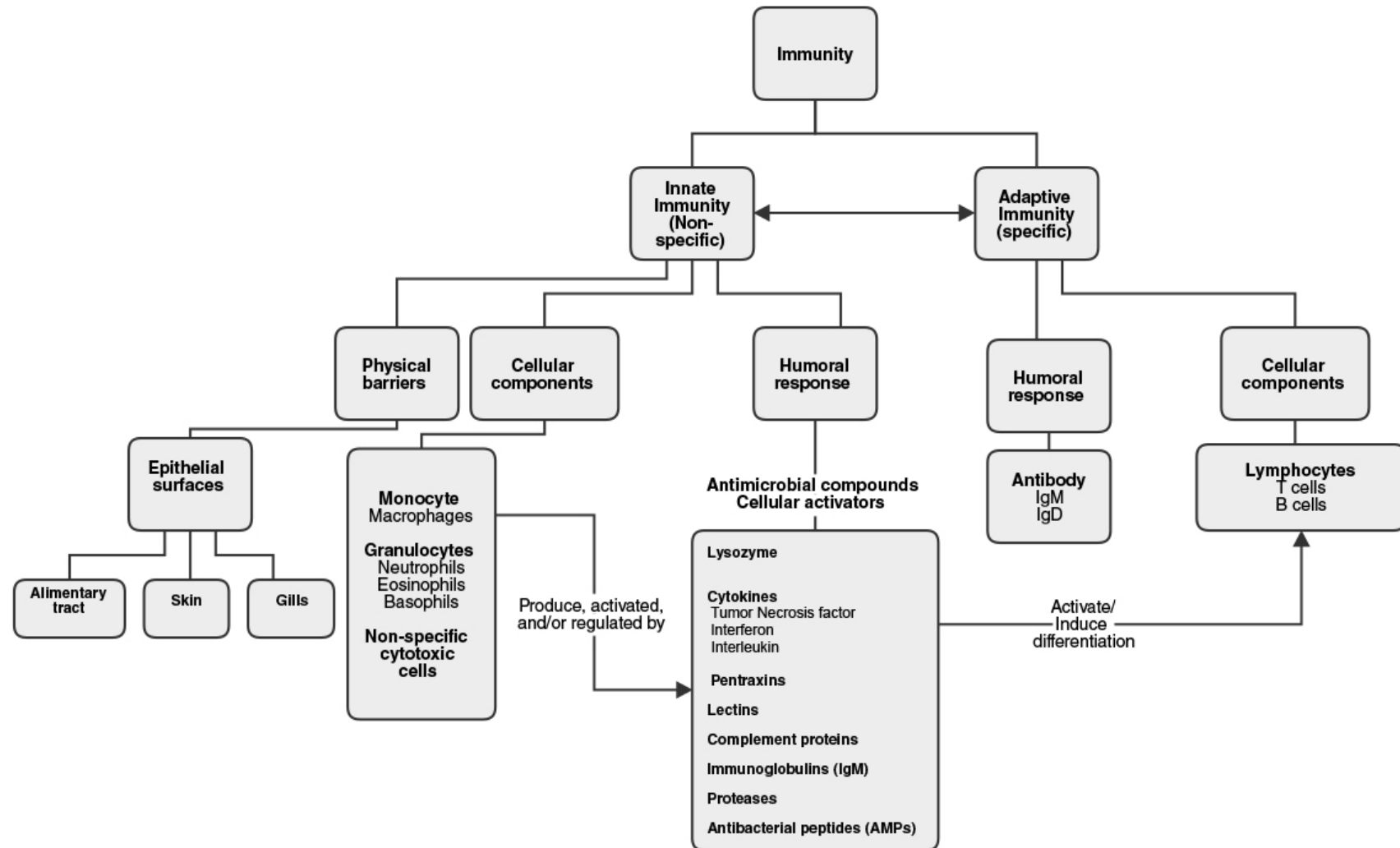


Figure 1. Summary of immunity in teleost fish.

1.1 Lymphoid system in fish

The lymphoid system in fish contains a variety of tissues and organs which play an important role in both the adaptive and innate immune response of teleost. Fish lymphoid organs and tissues include the thymus, interbranchial lymphoid tissue (ILT), kidney, liver, gut-associated lymphoid tissue (GALT), spleen, and skin-associated lymphoid tissue (SALT) (Figure 2).

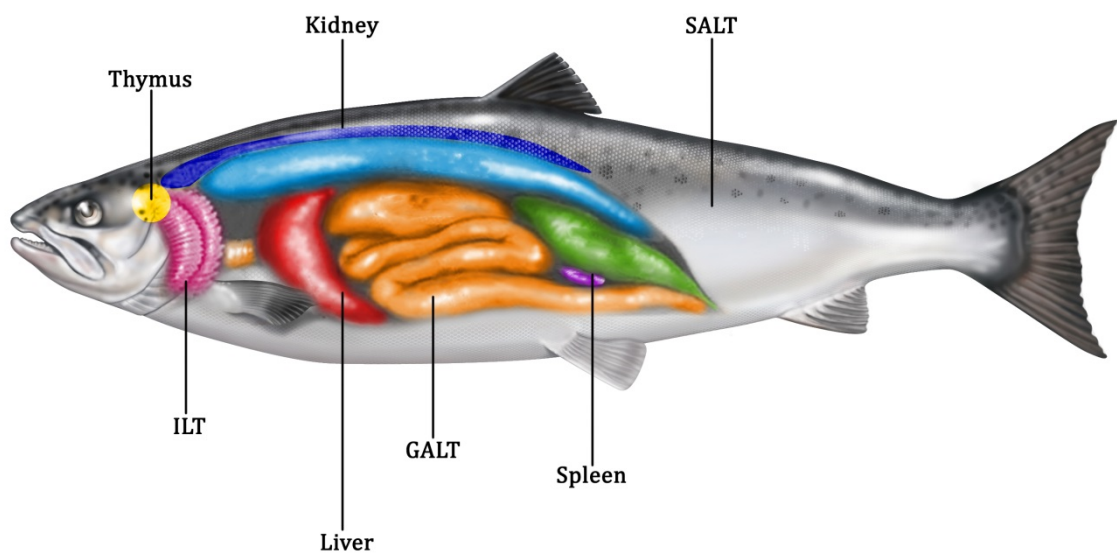


Figure 2. Salmonid fish highlighting the distribution of lymphoid organs and tissue based on images composited from Animal Life (<http://animalia-life.com/>).

The focus of this thesis is the non-specific immune response, primarily the interplay between cell-mediated immunity and humoral immunity. Therefore, these tissues are important because they are immunological sites and physical conduits for a variety of cells associated with innate immunity including monocytes, macrophages, neutrophils and granulocytes [5].

1.2 Components of the innate immune system in fish

The innate immune system represents the first and primary line of defence against pathogens in fish. It is an evolutionarily conserved and fundamentally important defense mechanism in fish. It can be split up into physical and chemical barriers (epithelial and mucosal), cellular components, and humoral responses [5]. The epithelial surfaces and mucosal barrier of the skin, gills, and alimentary tract act as physical barriers against pathogen invasion in fish [6]. While the epidermis and gill epithelium act primarily as an interface to the external environment, they also provide a substrate for antimicrobial activity. Mucus prevents pathogen adherence due to its continuous production and subsequent sloughing [7]. Additionally, mucus serves as a chemical storehouse for a wide variety of important innate immune components including lysozyme, lectins, immunoglobulins, proteases, and antibacterial peptides [7-9]. These secretory components are involved in direct pathogen destruction through a diverse range of antibacterial mechanisms, opsonisation of particles, complement activation, and the elicitation of a pro- or anti-inflammatory response [2, 10, 11].

The innate immune system relies heavily on pattern recognition receptors (PRRs) to distinguish between self and non-self and recognize pathogens [12-14]. These proteins are expressed by cells of the immune system and function through recognition of pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). Common bacterial PAMPs include lipopolysaccharide (LPS), mannose, bacterial DNA/RNA, flagellin, peptidoglycans, and lipoteichoic acids [3]. The four main PRRs which have been described in fish include Toll-like receptors (TLRs), NOD-like receptors (NLR), C-type lectin receptors (CLRs) and peptidoglycan recognition proteins (PGRPs) which can be classified according to their function or association with a particular ligand [12, 15]. Of these, TLRs are considered to be the best understood and 17 types have been recognized in fish [15]. Upon activation of TLRs, an innate humoral response is elicited. Molecules associated with this response include proinflammatory cytokines and type I interferons

(IFNs) which are important in modulating innate and adaptive immunity, as well as direct antimicrobial compounds such as antimicrobial peptides [12, 14].

1.3 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are amphipathic peptides which play an important role in innate defense [16-18]. They have been isolated from a wide variety of organisms including insects [19], amphibians [20, 21], mammals [22], bacteria [23], and fish [18]. They are commonly defined as having 12-50 amino acids, of which 50% or greater are hydrophobic, and an overall positive net charge [17]. AMPs generally require proteolytic cleavage to produce a mature peptide [24]. These peptides are gene-encoded and either constitutively expressed or upregulated during an infection. Additionally, transcriptional regulation of AMPs is often species specific and dependent on cell type [24, 25]. The expression of these peptides by immune cells usually works in conjunction with other components of innate immune response and is commonly associated with inflammation [22, 26, 27]. The mature peptides, upon contact with cellular membranes, fold into a variety of structurally diverse groups including: α -helical, β -sheet, cysteine rich and rare amino acid specific peptides [24, 25, 28]. While homology exists amongst the structural classification of AMPs, peptides exhibit high variability between sequences which is often associated with the multifactorial roles of these peptides, as well as the variety of highly specific pathogens each host organism encounters [29, 30]. In an effort to better characterize these differences and aid in new therapeutic discovery, a variety of antimicrobial databases have been established [31-33].

The broad-spectrum activity of AMPs against a wide range of microorganisms including Gram positive and Gram negative bacteria, protozoa, fungi, and viruses is well documented [22, 24, 27-29, 34-40]. Most active AMPs are capable of interacting with bacterial membranes through electrostatic and/or hydrophobic interactions, described by four known models [27]. The main direct antimicrobial functions are membrane disruption [30] and targeting intracellular components of

pathogens [41, 42]. It is generally accepted that positively charged peptides interact directly with the negatively charged cellular membranes of bacterial cells through the creation of ion-permeable channels, resulting in increase of membrane permeability, which leads to a rapid cell death. It is important to note that while AMPs have exhibited a tremendous ability to act as direct antimicrobial agents; many limitations to their use do exist. Primarily, AMPs are antagonized by monovalent and divalent cations which are commonly present in whole blood or serum [43, 44]. Many of the peptides which exhibit low molar concentration killing of bacteria *in vitro* require selective media with low salt concentrations to be effective [45, 46]. Additionally, some naturally produced AMPs have shown haemolytic activity towards host cells at concentrations required to be effective against pathogens. This is commonly linked to the hydrophobic nature of these peptides [47]. For these reasons, AMPs are commonly found in very low molar concentrations within their respective hosts. Moreover, while many of these peptides most certainly are bactericidal, *in vivo* evidence suggests a multifunctional purpose for some of these peptides [48].

Recently, many researchers have been focusing on the immunomodulatory abilities of AMPs at physiological concentrations and their influence on innate as well as adaptive immunity. These peptides are commonly called host defense peptides (HDPs) referring to their ability to enhance or modulate the host immune response to pathogens [24, 46, 48]. In some multicellular organisms, primarily mammals, HDPs have been shown to induce both pro- and anti-inflammatory cytokine production [49-52], act as chemoattractants of immune cells [36, 51, 53, 54], bind and inhibit bacterial LPS function [27, 55, 56], and facilitate the transfer of extracellular DNA/RNA across cellular membranes [52, 57-59]. Moreover, the antimicrobial properties of HDPs can be considerably affected by the salts and other cellular components present in the blood, organs, mucus, and other body fluids [36, 60, 61]. While many AMPs are antagonized by similar conditions, HDPs are unique because they are still able to confer protection *in vivo*, suggesting that while their direct antimicrobial activity is mostly lost, their broad range of immunomodulatory activities remain active [46, 62-64].

1.4 AMPs in fish

Fish are continually exposed to aquatic pathogens and secrete a wide range of AMPs as a primary defense mechanism. Teleost fish express a number of AMPs in mucus and blood as well as a variety of organs that are important for immune defenses including kidney, spleen, intestine, gills, reproductive organs and eyes [16-18, 34, 37, 39]. Additionally, many important AMPs have been documented in aquaculturally-relevant species (Table 1).

While some fish AMPs are constitutively expressed, others are up-regulated during bacterial infections. For instance, research involving the Atlantic salmon cathelicidins (asCATH1 and asCATH2) has shown that asCATH2 was constitutively expressed in a range of organs, including anterior and posterior kidney, sampled from healthy Atlantic salmon while asCATH1 was not found to be expressed in any tissues [65]. Additionally, fish cathelicidin expression studies show that CATH2 cathelicidins are widely expressed whereas CATH1 cathelicidins are more restricted and often induced by bacterial components [65-67]. Similarly, other AMPs have been shown to be inducible by bacterial challenge or bacterially-derived components [65, 68-73]. The increased expression of AMPs after bacterial induction highlights the important role these peptides play as a primary defense mechanism in fish innate immunity.

Table 1. A list of some AMPs from important aquaculture species and their putative functions.

Source organism	Peptide	Notes	Ref.
Atlantic cod (<i>Gadus morhua</i>)	CodCath	Cathelicidin; Isolated from kidney/spleen; Antimicrobial activity against Gram positive/ Gram negative bacteria and fungi; Activity is salt sensitive.	[74]
Atlantic cod (<i>Gadus morhua</i>)	Pis1	Piscidin; Antimicrobial activity against both Gram positive and Gram negative bacteria.	[75]
Atlantic cod (<i>Gadus morhua</i>)	Pis2	Piscidin; Antimicrobial activity against Gram negative bacteria.	[75]
Atlantic cod (<i>Gadus morhua</i>)	defB	β -defensin; Antimicrobial activity against Gram positive bacteria and stimulatory effect on phagocytic activity.	[76]
Atlantic salmon (<i>Salmo salar</i>)	asCATH1	Cathelicidin; Antimicrobial activity against both Gram positive and Gram negative bacteria. Increased transient expression of IL-8 in PBL <i>in vitro</i> .	[65, 66]
Atlantic salmon (<i>Salmo salar</i>)	asCATH2	Cathelicidin; Antimicrobial activity against both Gram positive and Gram negative bacteria. Increased transient expression of IL-8 in PBL <i>in vitro</i> .	[65, 66]
Atlantic salmon (<i>Salmo salar</i>)	SAMP H1	Peptide derived from histone H1; Expressed in skin mucus as antibacterial agent.	[77, 78]
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Oncorhyncin II	C-terminal fragment of histone H1; Antimicrobial activity against both Gram positive and Gram negative bacteria. Potentially important role in mucosal defence.	[79]
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Oncorhyncin III	Cleavage product of the non-histone chromosomal protein H6; Antimicrobial activity against both Gram positive and Gram negative bacteria. Potentially important role in mucosal defence.	[80]
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Hepcidin	Hepcidin; role in iron homeostasis during inflammation as well as acting as an antimicrobial peptide	[81]
Rainbow trout (<i>Oncorhynchus mykiss</i>)	omDB-2	β -defensin; Induced in skin, gills, and gut tissue after <i>in vivo</i> bacterial challenge. Upregulated after head kidney primary leucocyte cultures were stimulated with (polyI:C) <i>in vitro</i> .	[68]
Rainbow trout (<i>Oncorhynchus</i>	omDB-3	β -defensin; Induced in skin, gills, and gut tissue after <i>in vivo</i> bacterial challenge. Upregulated after head kidney primary leucocyte cultures were stimulated with (polyI:C) <i>in vitro</i> .	[68]

<i>mykiss</i>)			
Rainbow trout (<i>Oncorhynchus mykiss</i>)	omDB-4	β -defensin; Induced in skin, gills, and gut tissue after <i>in vivo</i> bacterial challenge. Upregulated after head kidney primary leucocyte cultures were stimulated with (polyI:C) <i>in vitro</i> .	[68]
Tilapia (<i>Oreochromis mossambicus</i>)	TH1-5	Hepcidin; Induced by (polyI:C) in liver and head kidney tissue after <i>in vivo</i> after <i>in vivo</i> i.p injection.	[82]
Tilapia (<i>Oreochromis mossambicus</i>)	TH2-2	Hepcidin; Induced in head kidney tissue after <i>in vivo</i> i.p injection. No noted antimicrobial ability.	[82]
Tilapia (<i>Oreochromis mossambicus</i>)	TH2-3	Hepcidin; Induced by LPS in liver tissue after <i>in vivo</i> i.p injection.	[82]

The direct antimicrobial and bactericidal functions of fish AMPs is well documented [18, 65, 66, 72, 75, 83-85], with most showing modest *in vitro* inhibition of both Gram negative and Gram positive strains. Like their mammalian counterparts, it is generally accepted that the positively charged nature of fish AMPs facilitates binding to negatively charged molecules or components in bacterial membranes and cause direct lysis through pore formation or inactivation of biological properties associated with the membranes. Piscidin, an antibacterial peptide isolated from the mast cells of hybrid striped bass (*Morone saxatilis* x *M. chrysops*) has been shown to remain unstructured in water but had high α -helix content in dodecyl-phosphocholine (DPC) micelle which mimics bacterial membranes. Additional multichannel and single channel experiments were used to show that this peptides act through the permeabilization of the bacterial membrane via pore formation [86]. This non-specific but highly functional mechanism for membrane disruption allows AMPs to destroy microbes at micromolar concentrations. Moreover, some fish AMPs have shown antiviral and antifungal properties. Tilapia (*Oreochromis mossambicus*) hepcidin 1-5 (TH 1-5) exhibited antiviral activities against nervous necrosis virus (NNV) *in vitro* [87]. Epinecidin-1, an AMP isolated from orange-spotted grouper (*Epinephelus coioides*), along with TH 1-5 were also shown to significantly decrease grouper larvae mortality after co-injection with NNV. Furthermore, re-challenge with the virus after 30 days in the co-treated group showed high survival [88]. Piscidin-2 (P2) isolated from hybrid striped bass (*Morone saxatilis* x *M. chrysops*) exhibited potent antifungal activity and caused fungal membrane damage against human pathogenic fungi *in vitro* [89]. This has led to discussion of its potential application in human dermatology [16].

In addition to their anti-infective capabilities, some fish AMPs possess immunomodulatory properties similar to their mammalian counterparts. Previous work by our group has demonstrated that Atlantic salmon cathelicidins (asCATH1 and asCATH2) stimulate the transient expression of the IL-8, an important chemotactic chemokine, *in vitro* in peripheral blood leukocytes [65]. Tilapia hepcidin (TH2-3) was shown to down-regulate the proinflammatory cytokines TNF- α , interleukin (IL)-1 α , IL-1 β , IL-6, and the prostaglandin synthesis gene, cyclooxygenase (COX)-2 in LPS-stimulated mouse macrophages [90] and modulate protein kinase C (PKC)-associated proteins in RAW264.7 macrophages [91]. In both studies, the powerful ability of TH2-3 to suppress LPS-induced proinflammatory cytokines was shown. Zebrafish (*Danio rerio*) β -defensin 2 (zfBD2) coupled with a plasmid delivery system was shown to trigger the activation of the type 1 IFN-system, induce the transcription of TNF- α and IL-1 β which are both involved in inflammation, increased the presence of major histocompatibility complex class II (MHC-II) transcripts, enhanced the cytotoxic cell response, and mediated the recruitment of Th cells at the injection site [92]. In contrast, the plasmid alone did not elicit these responses. Additionally, epinecidin-1 was shown to modulate the expression of immune-responsive genes, decreased TNF- α production, and significantly increased the survival rate of mice *in vivo* [93]. These mammalian studies provide preliminary evidence that fish AMPs are capable of modulating immune responses in higher vertebrates. Furthermore, they exhibit the potential of fish AMPs as more than mere antimicrobial compounds but also as immunostimulants and potential adjuvants.

Despite the many positive aspects of natural AMPs, many barriers still exist in their road to therapeutic application. The development of these peptides as systemic therapeutics has

been hampered by their potential for toxic side effects (cytotoxicity) and liability to proteolytic degradation [29, 43, 46]. The high antibacterial activity of natural AMPs *in vitro* is often correlated with increased haemolytic activity, or the ability to lyse eukaryotic cells [48, 94]. Moreover, the antimicrobial activity of AMPs is antagonized to variable extents by cellular and physiological components (cationic salts) in mucus, blood, and tissue [24, 28, 29, 45, 46]. Consequently, most clinical trials to date have focused on AMPs for topical applications. Furthermore, a major deterrent to natural peptide production is the cost associated with their production and the purity required for their therapeutic application. Currently, solid-phase chemical synthesis and to a lesser extent recombinant methods are used to produce AMPs synthetically [24, 95, 96]. These methods favour short peptides with relatively simple structural features. However, many natural AMPs are quite long (12-50 amino acids), have diverse structural components such as several disulphide bonds which are difficult to recreate through chemical synthesis, and many require post-transcriptional modification to produce functional mature peptides [43, 96, 97].

1.5 Rationale for synthetic peptide selection

In recent years, improved peptide synthesis methods and large, continually updated AMP databases have allowed for the production of synthetic AMPs with many superior characteristics to their parent peptides [31-33, 98]. The parameters, methodology, and approaches used to conceptualize and develop synthetic peptides are extremely diverse and entirely dependent on the intended application of the final peptide (Figure 3). Moreover, these methods are rapidly evolving in stride with new production technology. General approaches adopted in the design of synthetic AMPs include sequence modifications from naturally occurring template peptides, *de novo* minimalist design of amphipathic peptides,

and the use of computer-assisted virtual screening to aid in the identification of new lead sequences [97, 99]. The majority of the peptides used in this thesis were created using these methodologies. Additionally, many of the peptides also possess the potent properties of cationic cell-penetrating peptides. P9-4 was developed from the Trp-rich peptide Pac-525 and P11-5/ P11-6 were developed using BP76 as a template [100, 101]. These peptides exhibit improved stability in the presence of salts, reduced cytotoxicity and substantially improved MICs compared to their parent peptides [102]. K₆L₂W₃ and K₇LW₃ were also designed based on a Trp-rich peptide, Indolicidin [103]. These peptides showed high cell specificity towards bacterial cells in a previous study. Moreover, K₆L₂W₃ exhibited anti-inflammatory activity by inhibited LPS-induced nitric oxide synthase (iNOS) mRNA expression and the release of nitric oxide (NO) following LPS stimulation in RAW264.7 cells *in vitro* [103]. Pep-1K was modelled after the cell-penetrating peptide Pep-1 and showed improved MICs compared to the reference peptide [104]. PG-1 was derived from porcine leukocytes and represents the only β -sheet peptide evaluated in this thesis. Previous studies involving PG-1 showed its ability to neutralize the effect of the *Neisseria meningitidis* endotoxin through significantly decreased TNF- α release from THP-1 cells and nitric oxide release from RAW 264.7 cells [50], as well as its potent antimicrobial capabilities *in vitro* and *in vivo* efficacy in mice trials [105].

However, HHC-10 was developed using an entirely different method than the previous mentioned peptides. HHC-10 was the product of advanced *in silico* predictive modelling and artificial intelligence [106]. In this study, Cherkasov and his colleagues used a chemo-informatics approach called quantitative structure-activity relationship (QSAR) and Artificial Neural Networks, a powerful machine learning technique, to assess and predict the antibiotic activity of 100,000 virtual peptides based on two large random 9-amino-acid

peptide libraries. HHC-10, and one other peptide, represent the top quartile of predicted activities and were selected to undergo additional testing. The results of this study showed that HHC-10 was effective against many strains of multidrug resistant bacteria with activities that equal or outperform conventional antibiotics. Additionally, it was shown to protect against *Staphylococcus aureus* infections in animal models [106].

Taken as a whole, all of these cationic synthetic peptides represent novel and interesting candidates for therapeutic applications in fish-related disease. In Chapters 2 & 4 of this thesis, the potential use of these peptides is explored in greater depth.

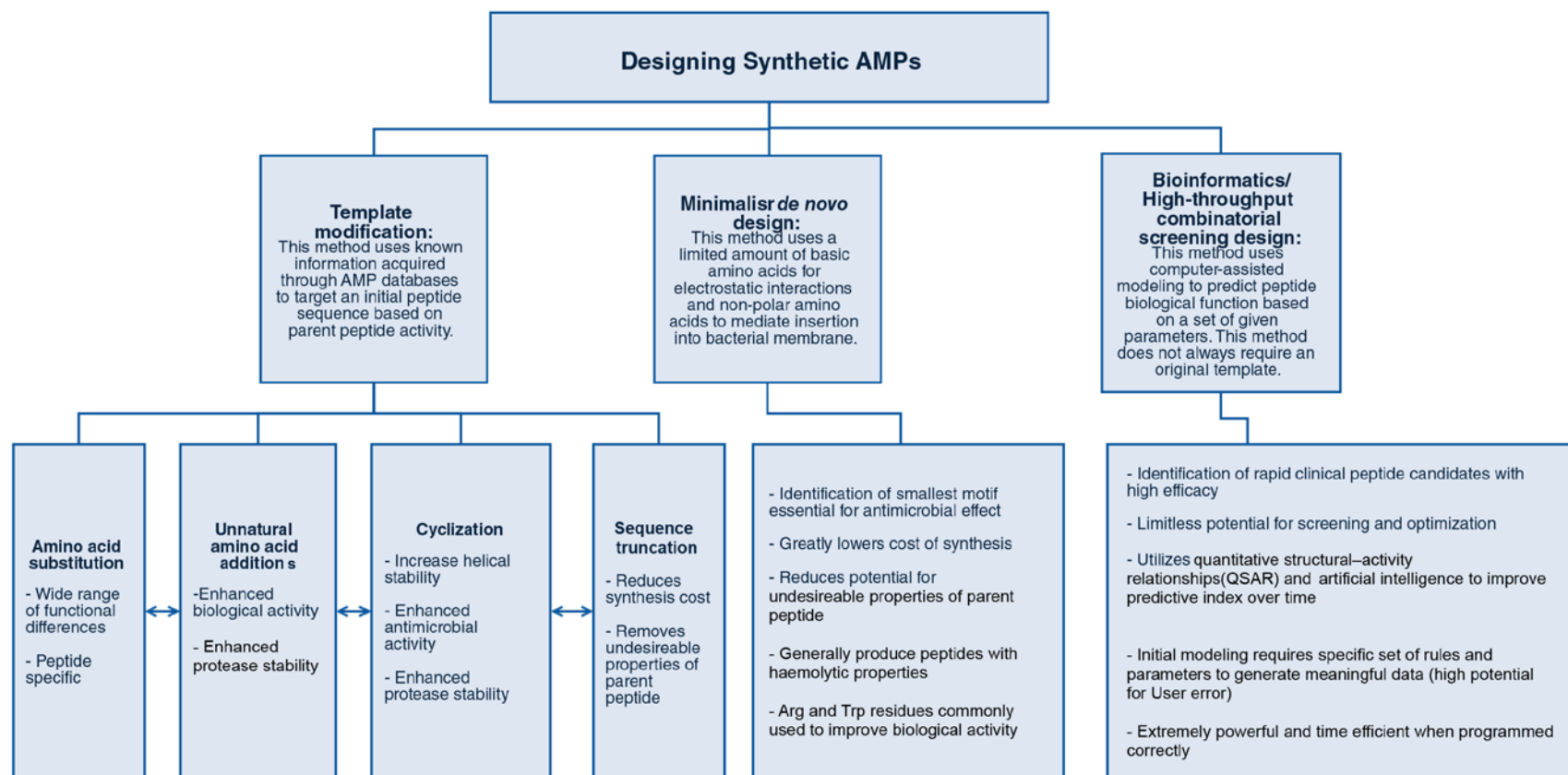


Figure 3. Overview of strategies employed in the design and optimization of synthetic AMPs.

1.6 Thesis aims

The major aim of this research is to investigate the dynamic role AMPs play in the innate immune system of fish, both as direct antimicrobial agents and in their ability to modulate immune response.

Research in the following chapters has been focused on the following key objectives:

- Investigate the potential immunomodulatory capabilities of Atlantic salmon cathelicidins (asCATH1 and asCATH2) on *S. salar* leukocyte populations (Chapter 2)
- Investigate the potential therapeutic applications of novel synthetic cationic peptides on *S. salar* varying leukocyte populations
 - Assess the direct antimicrobial capabilities of synthetic peptides against a range of aquatic pathogens *in vitro* and establish MIC for each (Chapter 3)
 - Assess the immunostimulatory and immunomodulatory capability of synthetic peptides and effect on *S. salar* leukocyte populations (Chapter 3 and 4)

Chapter 2: Atlantic salmon cathelicidin (asCATH2) increases phagocytosis, cell proliferation and respiratory burst in Atlantic salmon (*Salmo salar* L.) head kidney leukocytes

2.1 Introduction

Cathelicidins are a class of AMPs (AMPS) which play an important and multifunctional role in the innate immune system of multicellular organism [26, 28, 35]. These host defense peptides consist of a homologous N-terminal signal sequence and proregion, a cathelin-like domain from which their name is derived, and a variable C-terminal antimicrobial domain [107]. Peptides are stored in immune cells as inactive precursors (prepropeptides) which undergo proteolytic cleavage during an infection to release a mature peptide with cationic and hydrophobic attributes [26, 107]. While the resultant antimicrobial peptide is highly variable in size and sequence composition even within species, all peptides have an overall positive net charge [17, 26, 65, 107]. These amphipathic peptides function as natural antibiotics produced by the host, disrupting the cell membrane of invading bacteria during an infection [65, 66]. They are able to combat a wide variety of pathogens including Gram negative and Gram positive bacteria, parasites, fungi, and viruses [28]. While the mammalian cathelicidins are the most widely studied, this class of peptides exists in a wide variety of insects [22], reptiles [108], and fish [66, 107, 109] with new organisms constantly being added to the list.

In addition to their antimicrobial capacity, cathelicidins work in conjunction with other inflammatory cells and possess a diverse range of immunomodulatory features. These features include the ability to recruit host immune cells to the site of infection [22, 27, 36, 48], stimulate or inhibit cytokines and chemokines from a variety of different cell types [24, 36, 110], and alter host gene expression [65, 111-114]. More recently, cathelicidins have been shown to bind and facilitate the uptake of extracellular components into host immune cells during prolonged infections [57, 59, 115]. These functions allow for rapid identification

and destruction of pathogens and highlight the multifaceted role of AMPs in the innate immune system.

While a great deal of information can be found concerning the role of cathelicidins in mammals [26, 28, 116], cathelicidins have only recently been identified and characterized in fish [26, 66, 74, 109]. Many of the species which have been characterized are commercially relevant and include rainbow trout (*Oncorhynchus mykiss*) [109], Atlantic salmon (*Salmo salar*) [66], Atlantic cod (*Gadus morhua*) [67, 107], and Arctic charr (*Salvelinus alpinus*) [74, 107]. Previously, our group explored the role of these peptides during an infection with *Y. ruckeri*. AsCATH2 was shown to be constitutively expressed in healthy Atlantic salmon while asCATH1 was not, and the expression of both was induced following bacterial challenge. Additionally, it was demonstrated that Atlantic salmon cathelicidins stimulated the expression of chemokine interleukin-8 in peripheral blood leukocytes (PBLs) [65]. Recent evidence has suggested that cathelicidins in mammals have the ability to promote phagocytosis by leukocytes [64]. It remains unclear if this is a function limited to mammalian cathelicidins or to the class of peptides as a whole.

The aim of this study was to assess the *in vitro* immunomodulatory ability of asCATH1 and asCATH2 using head kidney leukocytes in Atlantic salmon (*Salmo salar*). Functional assays were utilized to assess the direct stimulatory capacity of these peptides on phagocytosis, cell proliferation, and respiratory burst.

2.2 Materials and Methods

2.2.1 Ethics statement

All work involving animals was approved by the University of Tasmania Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2.2 Reagents

Oligodeoxynucleotides purchased from Sigma Genosys (Castle Hill, NSW, Australia) were phosphorothioated to increase their resistance to nuclease degradation [117]. The sequence for CpG-ODN 1668 was (TCCATGACGTTCTGATGCT). This Oligodeoxynucleotide sequence was selected based on its effective use in Atlantic salmon, rainbow trout, other teleost fish, and murine studies [118-121].

The two salmonid peptides (asCATH1 and asCATH2) were chemically synthesised by Auspep (Tullamarine, Victoria, Australia). These peptides corresponded to the first 36 amino acids of each mature peptide of the previously reported Atlantic salmon cathelicidins [66]. The first peptide, referred to as asCATH1 (RRSQARKCSRGNNGGKIGSIRCRGGGTRLGGGSLIGR), had a molecular weight of 3685 g/mol and corresponds to residues 145 to 180 of the *S. salar* cathelicidin (acc. #: AAW55907). The second, referred to as asCATH2 (RRGKPSGGSRGSKMGSKDSKGGWRGPGSGSRPGFG), had a molecular weight of 3632 g/mol and corresponds to residues 150 to 185 of the *S. salar* cathelicidin-derived antimicrobial peptide 2 precursor (acc. #: AAT44537). These cathelicidin derived peptides and their corresponding concentrations were selected based on their suggested antimicrobial and immunomodulatory role in a previous study [65].

2.2.3 Fish

Atlantic salmon weighing approximately 200-300 g were maintained in a 3000L freshwater water re-circulating tank with biofilter at the School of Aquaculture, University of Tasmania, Launceston, Australia. Fish were euthanized with a lethal dose of anaesthetic at 5 g/L AQUI-S® (AQUI-S NZ Ltd, Lower Hutt, New Zealand) prior to head kidney isolation.

2.2.4 Isolation of head kidney leukocytes

Head kidney leukocytes were isolated as previously described [122]. Briefly, head kidney was aseptically removed from each fish and repeatedly passed through a 100 µM sterilized metal mesh using L-15 medium containing 1% penicillin and streptomycin (P/S) (Sigma), 0.2% heparin (20 U/mL) (Sigma). The resulting cell suspensions were placed on a 34/54% Percoll (Sigma) gradient and centrifuged at 400 g for 40 min at 4°C. The interface was harvested and washed twice in phosphate buffered solution (PBS, Sigma) at 400 g for 5 min in previously mentioned media. Cells were quantified using a haemocytometer by trypan blue exclusion for use in their respective assays.

2.2.5 Phagocytosis assay

Phagocytosis was assessed using methods previously described with modification [123]. Briefly, cells were seeded in 96-well plates at a density of 1×10^7 cells per well, and allowed to adhere at 18°C for 3 h. Wells were then washed three times with L-15 medium containing 1% penicillin and streptomycin to remove any non-adherent cells. Cells were stimulated with asCATH1 (5µM), asCATH2 (5µM), or CpG-ODN 1668 (5µM) for 4 h at 18°C. Control leukocytes were incubated with L-15 medium alone. After the incubation period, 20 µL Congo red-stained yeast cells (10^7 cells/mL) were added to each well. To monitor

phagocytosis, two pictures were taken randomly for each well using a Cannon 600D Digital SLR with an optical adapter on a light microscope at given time points. The phagocytic activity (PA) was determined by the percentage of cells with engulfed yeast cells (PA = number of phagocytic cells with engulfed bacteria/number of phagocytes). The phagocytic index (PI) was determined by the number of engulfed yeast cells per cell (PI = number of engulfed yeast/phagocytic cells).

2.2.6 *Respiratory burst activity assay*

Respiratory burst activity was assessed by nitro blue tetrazolium (NBT) (Sigma) reduction to formazan after stimulation of cells as previously described [123]. Briefly, cells were isolated and stimulated for 4h at 18°C in 96-well plates as stated above. Untreated leukocytes were used as the negative control and leukocytes treated with phorbol myristate acetate (PMA) (100 ng/mL) as a positive control. After stimulation, the cell monolayer was washed twice with PBS to remove any residual stimulant and 100 µL of NBT solution (1 mg/mL in L-15) was added per well. Cultures were then incubated at 18°C for 1 h. After incubation, cells were fixed with methanol after the removal of the NBT solution. Cells were then air dried for 20 min then washed again to remove residual methanol. Finally, 120 µL 2M KOH and 140 µL of DMSO (Sigma) were added to each well and the optical density was measured with an Infinite M200 Pro microplate reader (Tecan) at 620 nm.

2.2.7 *Cell proliferation assay*

Cell proliferation was assessed by measuring total nucleic acid content of lysed cells after stimulation treatments using a fluorometer. Briefly, cells were seeded in 96-well plates at a density of 1×10^5 cells per well, and allowed to adhere at 18°C for 3 h. Wells were then

washed three times with L-15 medium containing 1% penicillin and streptomycin and 10% to remove any non-adherent cells. Cells were stimulated with asCATH1 (5 μ M), asCATH2 (5 μ M), or CpG-ODN 1668 (5 μ M) for 5 days at 18°C. Control leukocytes were incubated with L-15 medium alone. Cells were immediately frozen after stimulation. Total nucleic acid was extracted as previously described with modification [124]. Briefly, previously stimulated 96-well plates were defrosted and 300 μ L nucleic acid extraction buffer (4M urea, 1% SDS, 0.2 M NaCl, 1 mM sodium citrate pH 7.5) containing 20 U of proteinase K (Bioline) per sample was added to each well. 96-well plates were then placed on ice and allowed to digest for 30 min. Samples were transferred from their wells into fresh 1.5 mL microcentrifuge tubes. Protein, cellular debris, and detergent were removed by centrifugation in 7.5M ammonium acetate at 14,000 x g for 5 min, and nucleic acids were recovered by isopropanol precipitation at 16,000 x g for 10 min followed by an ethanol wash of the nucleic acid pellet. Complete removal of RNA was ensured by treatment with 4 units RNase A (Sigma) for 30 min at 37°C. Samples were then resuspended in molecular water and quantified using a Qubit fluorometer (Invitrogen) using the manufacturer's protocol.

2.2.8 Statistical analysis

Statistical analysis was carried out using GRAPHPAD™ PRISM version 5.00 for WINDOWS® (GraphPad Software). Differences between groups were determined using a one-way ANOVA with a Tukey posthoc test. Data were subject to a Shapiro–Wilk's test for normality and a Levene's test for equality of variances to satisfy the assumptions required for an ANOVA. Results were considered significant if $P < 0.05$.

2.3 Results

2.3.1 Phagocytosis assay

Phagocytic activity of head kidney leukocytes stimulated with asCATH1 (5 μ M), asCATH2 (5 μ M), CpG-ODN 1668 (5 μ M) or an untreated cell control differed significantly ($F_{[3, 8]} = 36.06$, $P < 0.0001$; Figure 4A). A Tukey's post-hoc analysis of the groups indicated that there was a significant increase in the phagocytic activity of asCATH2 compared to the control and asCATH1 ($P < 0.05$). In addition CpG-ODN 1668 had a significantly higher phagocytic activity than all other treatments ($P < 0.05$). There was no significant effect on phagocytic index by any of the treatments ($F_{[3, 8]} = 0.6703$, $P = 0.5938$; Figure 4B).

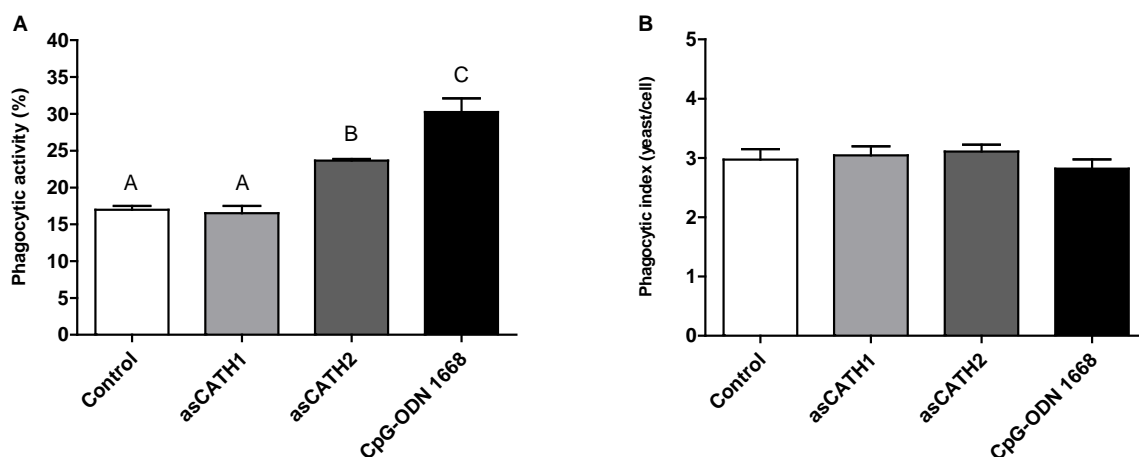


Figure 4. Phagocytic activity (a) and phagocytic index (b) of Atlantic salmon (*S. salar*) head kidney leukocytes stimulated with asCATH1 (5 μ M), asCATH2 (5 μ M), or CpG-ODN 1668 (5 μ M). The phagocytic activity (PA) was determined by the percentage of cells with engulfed yeast cells (PA = percentage of phagocytic cells with engulfed bacteria). The phagocytic index (PI) is determined by the number of engulfed yeast cells per cell (PI = number of engulfed yeast/phagocytic cells). Control leukocytes were incubated with medium alone. Data shown are the means \pm SE of quadruplicate HKL samples assayed from 3 fish. Different superscript letters indicate significant differences between the groups ($P < 0.05$).

2.3.2 Respiratory burst assay

Respiratory burst of head kidney leukocytes stimulated with asCATH1 (5 μ M), asCATH2 (5 μ M), CpG-ODN 1668 (5 μ M), PMA (100 ng/mL), or an untreated cell control differed significantly between treatments ($F_{[4, 10]} = 18.70$, $P = 0.0001$; Figure 5). A Tukey's post-hoc analysis of the groups indicated that there was a significant increase in the NBT reduction of asCATH2 compared to the control ($P < 0.05$). CpG-ODN 1668 had a significantly higher NBT reduction than all other treatments ($P < 0.05$) except the positive control, PMA, which had significantly greater NBT reduction than all other treatments ($P < 0.05$).

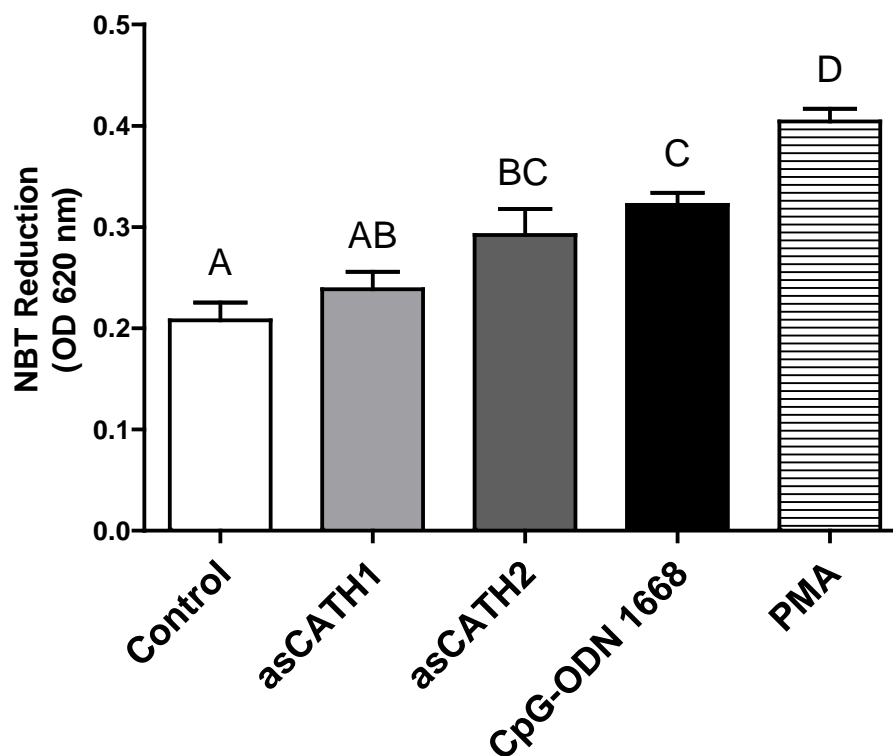


Figure 5. Respiratory burst activity of Atlantic salmon (*S. salar*) head kidney leukocytes stimulated with asCATH1 (5 μ M), asCATH2 (5 μ M), or CpG-ODN 1668 (5 μ M). Control leukocytes were incubated with medium alone and positive controls were treated with PMA (100 ng/ml). Data shown are the means \pm SE of quadruplicate HKL samples assayed from 3 fish at 620 nm after 60 min incubation. Different superscript letters indicate significant differences between the groups ($P < 0.05$).

2.3.3 Cell proliferation assay

Cell proliferation of head kidney leukocytes stimulated with asCATH1 (5 μ M), asCATH2 (5 μ M), CpG-ODN 1668 (5 μ M), or an untreated cell control differed significantly between treatments ($F_{[3, 18]} = 80.81$, $P < 0.0001$; Figure 6). A Tukey's post-hoc analysis of the groups indicated that there was a significant increase in total DNA yield of cells treated with asCATH2 compared to the control and asCATH1 ($P < 0.05$). Cells stimulated with CpG-ODN 1668 had a significantly higher total DNA yield than all other treatments ($P < 0.05$).

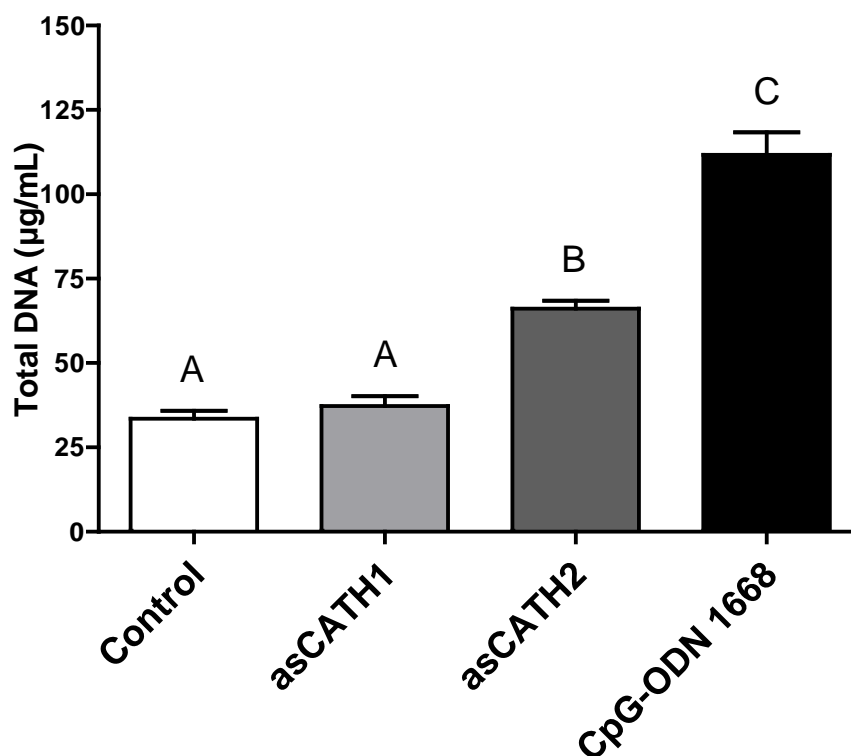


Figure 6. Cell proliferation of Atlantic salmon (*S. salar*) head kidney leukocytes stimulated with asCATH1 (5 μ M), asCATH2 (5 μ M), or CpG-ODN 1668 (5 μ M) for 5 days. Control leukocytes were incubated with medium alone. Data shown are the means \pm SE of quadruplicate HKL samples assayed from 3 fish. Different superscript letters indicate significant differences between the groups ($P < 0.05$).

2.4 Discussion

While the antimicrobial properties of cathelicidins are well described, recent research suggests that their ability to act as chemotactic agents [36] and immunomodulators [46, 48, 63, 125] may be the most relevant function in the context of bacterial infections. In this study, we demonstrated that the Atlantic salmon cathelicidin, asCATH2, was capable of improving leukocyte phagocytic activity and respiratory burst *in vitro*. Conversely, asCATH1 had minimal stimulatory activity *in vitro*. It has been well established that fish phagocytes possess oxidative burst responses comparable to those exhibited by mammalian phagocytes and that phagocytosis triggers the activation and rapid release of reactive oxygen species (ROS) via the NADPH oxidase complex [37]. Therefore, the increased phagocytic activity induced by asCATH2 correlates well with the improved respiratory burst seen when cells were stimulated with the same concentration of asCATH2. Moreover, research involving LL-37, the human cathelicidin, has shown it is capable of inducing a prophagocytic response at low concentrations of peptide but not at higher dosages despite a lack of cytotoxic activity on macrophages. In another study, LL-37 has been shown to induce the production of ROS in murine and human macrophages[50]. While the mature peptides of LL-37 and asCATH2 differ substantially in amino acid composition and structure, they are both able to elicit similar biological functions on host immune cells.

In addition to improved phagocytic function, asCATH2 induced increased cell proliferation in leukocytes stimulated for 5 days whereas asCATH1 did not vary significantly from the controls. Previous work by our group has demonstrated that Atlantic salmon cathelicidins stimulated the transient expression of the interleukin-8, an important

chemotactic chemokine, in peripheral blood leukocytes. In these studies, asCATH2 was found to be constitutively expressed in healthy fish and was upregulated to a greater extent than asCATH1 after bacterial challenge. Conversely, asCATH1 was not expressed in any organ, prior to bacterial challenge. This pattern of expression for CATH1 and CATH2 peptides has been shown to exist in other salmonids as well [66, 107, 126]. Additionally, asCATH2 showed no haemolysis towards erythrocytes in either serum or conventional media and lower antibacterial activity than asCATH1[65]. The upregulation during bacterial infections and minimized bactericidal activity may suggest that asCATH2 serves to modulate immune-competent cells during bacterial infections rather than function as a direct antimicrobial defense mechanism. Similarly, it has been suggested that LL-37 functions as an immunomodulator *in vivo* because it is produced constitutively at low concentrations (2–5 µg/ml) at mucosal surface and in most bodily fluids and lacks antimicrobial abilities at high concentrations (100 µg/ml) in tissue culture media[64]. Additionally, LL-37 has been shown to increase cell proliferation in human epithelial cell lines and promote wound healing [62, 127]. However, the question remains as to why asCATH2 is capable of immunomodulatory functions whereas asCATH1 is not. While an overwhelming amount of evidence suggests that AMPs interface directly with bacterial membranes to exhibit their antimicrobial action, this does not explain the diverse range of non-membrane mechanisms and alternative signaling cascades that some AMPs are able to elicit. Moreover, while difference in mature peptide structure exists between asCATH1 and asCATH2, amino acid composition alone is not a hallmark of bioactivity. A previous study suggests that the AMP membrane interactions cannot be explained by a particular sequential amino-acid pattern or motif but rather a combination of features, both structural and physicochemical [95].

Currently, information regarding the expression of these cathelicidins in salmonids has been isolated down to specific tissues but not specific cell types [65, 66, 107]. While the limited amount of information regarding AMPs, their cell-associated ligands, and immunological cascade signalling in salmonids makes it difficult to ascertain the specific functions of fish cathelicidin, the evolutionary status of teleost fish in relation to other vertebrates allows for speculation of potential cellular targets. In mammalian studies, cathelicidin expression has been closely related to TLR- activation [13, 128]. TLRs play an important role in recognizing specific microbial components derived from pathogens including bacteria, fungi, protozoa and viruses. These receptors recognize PAMPs through cell surface TLRs or through intracellular TLRs (Figure 7). Further, they play an important role in recognition of self and non-self and their signalling initiates a complex cascade of innate and adaptive immune responses.

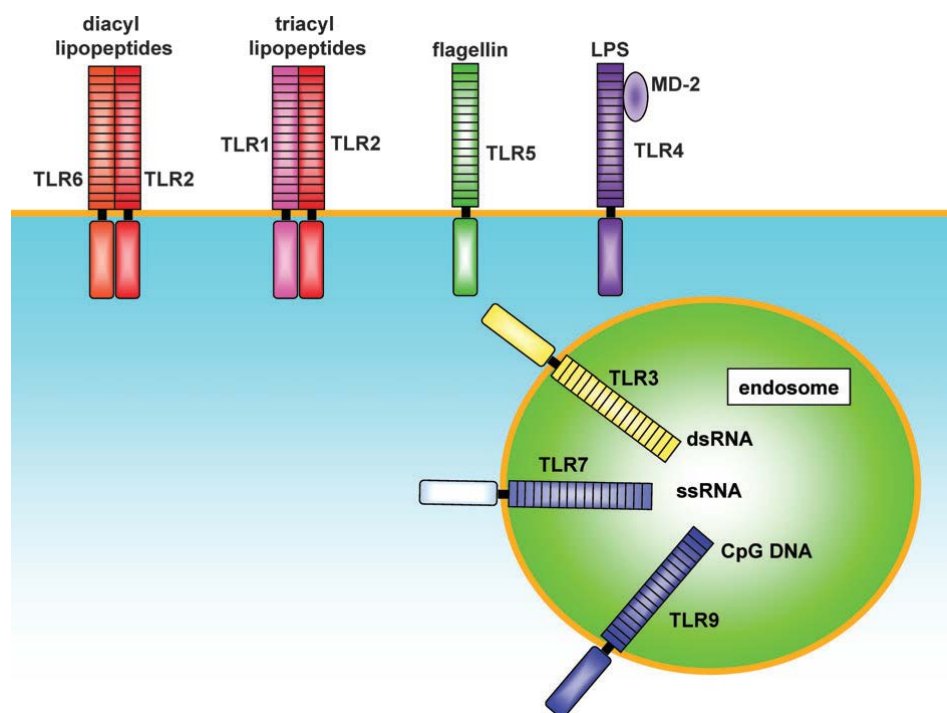


Figure 7. TLR family members recognize specific patterns of microbial components. Image source: [14].

Cathelicidins are known to both inhibit and activate inflammatory responses via TLR signaling. The bovine cathelicidin BMAP-28 is capable of suppressing the proinflammatory response induced by LPS in RAW 264.7 macrophages by blocking LPS-induced cytokine gene expression [129]. However, BMAP-28 alone was capable of inducing upregulation of IL-1 β gene expression in these cells, suggesting it has the capacity to activate selected cellular pathways through direct effects on macrophages [129]. The guinea pig α -helical cathelicidin CAP11 was shown to suppress the production of anandamide, a mediator of endotoxic shock, in LPS stimulated RAW 264.7 macrophages in a dose dependant manner [130]. Additionally, a previous study involving CAP11 showed that this peptide suppressed LPS-induced TNF- α mRNA and protein expression when analysed by Northern and Western blot [131]. Taken as a whole, these studies indicate that cathelicidins play an integral role in suppressing bacterially-derived inflammation through mitigation at the cellular level. Future work involving the salmonid cathelicidins could emulate these experiments to see if these peptides exhibit similar functions to other vertebrate cathelicidins. Studies could look into the differential expression of these peptides after the administration of a variety of different microbial compounds such as CpG-ODN, LPS, flagellin, and poly(I:C) in Atlantic salmon, as this has already been done *in vitro* in CHSE-214 cells [67]. Future studies should focus on identifying the specific pathways elicited by these peptides, establish whether or not this is dose-dependent, and screen a larger selection of immune-related genes than previous studies. These studies could provide invaluable insight into the role of cathelicidins during an innate immune response in salmonids.

In this study, CpG-ODN 1668 was utilized as a positive control due to its strong immunostimulatory ability. CpG-ODN 1668 exhibited the highest level of stimulation

significantly increasing phagocytosis, respiratory burst, and cell proliferation. CpG-ODN 1668 is a class B oligodeoxynucleotide containing a full phosphorothioate backbone and is known for the ability to strongly activate B cells in mammals [132]. Previous studies involving class B CpG-ODNs showed their ability to induce increased cell proliferation in Atlantic salmon head kidney leukocytes [133] and exhibit their immunostimulatory ability *in vivo* [119]. Furthermore, CpG-ODN has been shown to enhance phagocytic ability and ROS production by head kidney phagocytes when injected in carp (*Cyprinus carpio* L.) *in vivo* [134].

2.5 Conclusion

In conclusion, this study demonstrated that asCATH2 and CpG-ODN 1668 were capable of stimulating head kidney leukocytes to improve phagocytic activity, respiratory burst, and cell proliferation at micromolar concentrations while asCATH1 did not have any significant effect. This data further support the notion that asCATH2 may serve a multifaceted biological role during bacterial infections and that asCATH1 is induced after bacterial stimulation and primarily functions as a lytic peptide. Additionally, these data provide further evidence of the functional differences which exist between Atlantic salmon cathelicidins. Moreover, this study is believed to be the first to report that a fish cathelicidin is capable of increasing phagocytosis, cellular proliferation, and respiratory burst in head kidney leukocytes. While the mechanisms and stimulation pathways which govern this reaction remain unknown, these functional assays suggest that cathelicidins play an important role in bridging innate and adaptive immunity in salmonids.

2.6 Acknowledgements

We thank Philip Crosbie for his assistance in procuring the Atlantic salmon and the continued maintenance of the system in which they resided.

**Chapter 3: Short synthetic cationic peptides exhibit
antimicrobial activity and immunomodulatory functions *in
vitro* in Atlantic salmon (*Salmo salar*)**

3.1 Introduction

While natural sources of AMPs exist, synthetic analogs of these peptides have been created by the removal and or substitution of amino acids which correspond to peptide hydrophobicity and enhanced biological activities [44, 135]. Additionally, naturally occurring AMPs are larger in size and create several issues related to synthesis, metabolic stability and cost of production [136]. The purpose of this work is to characterise the antimicrobial and immunomodulatory properties of selected synthetic peptides (Table 2) against known fish pathogens in physiologically relevant environments. In previous trials comparing naturally produced AMPs, these synthetic peptides have shown high target specificity [103], low cytotoxicity to the host [102], low minimum inhibitory concentrations [102, 103], and improved stability in saline environments [102, 105].

3.2 Materials and Methods

3.2.1 *Ethics statement*

All work involving animals was approved by the University of Tasmania Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.2.2 *Fish Sampling*

Atlantic salmon (*Salmo salar*) were obtained from a recirculation tank-based population of fish maintained at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). Fish were euthanized with a lethal dose of anaesthetic at

1 g/L AQUI-S® (AQUI-S NZ Ltd, Lower Hutt, New Zealand) and blood was collected for serum and PBL assays.

3.2.3 Peptide synthesis

All peptides listed in Table 2 were synthesized using the solidphase method and standard 9-fluorenyl methoxy carbonyl chemistry and purified to >95% purity using reverse-phase high-pressure liquid chromatography by Life Research Australia. Certificates of analysis provided with each peptide displayed HPLC chromatogram and mass spectral analysis identifying the purity to be greater than 95% and identity of each peptide.

Table 2. Summary of synthetic AMPs used in this study.

Peptide	Sequence	Molecular weight (g/mol)	Purity (%)	Ref.
P9-4	Ac-KWRRWIRWL- NH2	1440.78	95.50	[102]
P11-5	Ac-GKLFKKILKIL- NH2	1341.80	96.89	[102]
P11-6	Ac-KKLIKILKIL- NH2	1378.91	96.97	[102]
HHC10	Ac-KRWKWKIRW- NH2	1485.82	99.27	[106]
K6L2W3	Ac-KLWKKWKKWLK-NH2	1613.09	96.84	[103]
K7LW3	Ac-KLWKKWKKWKK-NH2	1628.10	95.72	[103]
PG-1	Ac-RGGRLCYCRRRFCVCVGR-NH2	2201.68	96.54	[105]
Pep-1-K	Ac-KKTWWKTWWTKWSQPKKKRKV-NH2	2886.51	97.34	[34, 104]

3.2.4 Bacterial culture conditions

Aeromonas hydrophila, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa*, and the Tasmanian O1b serotype of *Yersinia ruckeri* (UTYR 001A) were used to test the antibacterial activities of the selected AMPs. *Aeromonas hydrophila* and *Yersinia ruckeri*

represent potential fish pathogens and *E. coli* and *P. aeruginosa* have known MICs for these peptides [102]. Bacteria were grown at 20°C in 4 mL of Mueller-Hinton broth (MHB) until they reached mid-log phase then 1 mL of the log-phase suspension was centrifuged at 1,000 xg for 5 minutes at room temperature. The supernatant was removed and the bacterial pellet was resuspended in 1 mL of one quarter strength (0.25x) MHB. The suspension was diluted 1:1000 by adding 10 mL to 10 mL of 0.25x MHB to give a final working suspension of approximately 1×10^5 cells/mL.

3.2.5 Antimicrobial and salt tolerance assay

A broth microdilution assay was used to measure the antimicrobial activity and salt tolerances of the selected synthetic peptides as previously described [137]. In brief, bacteria were grown to a mid-log phase in MHB and diluted to 1×10^5 cells/mL. Each peptide was serially diluted in sterile water to give final concentrations of 800, 400, 200, 100, 50, 25, 12.5 and 6.25 μ M. 90 μ L of the 1:1000 bacterial suspension was combined with 10 μ L of each peptide dilution in a flat bottom 96 well polystyrene microtitre plate so that final peptide concentrations were 80, 40, 20, 10, 5, 2.5, 1.25 and 0.63 μ M. To determine the resistance of the AMPs against salt, NaCl in varying concentrations (0-300 mM) was added to the samples. Each species had four technical replicates. Plates were incubated at 20°C for 18 h then absorbances were read at 600 nm. Positive controls contained bacterial suspension with 10 μ L of water and negative controls contained 0.25x MHB media with no bacterial cells. Microbial growth was determined by optical density measurement at 600 nm using a microplate reader (Tecan GENios). The MIC was defined as the lowest concentration of peptide that reduced bacterial growth by more than 50% compared to positive control wells [66, 109, 138].

The synthetic peptides P9-4, P11-5, P11-6, and PG-1 were chosen as candidates to examine interactions between peptide and serum using a serum microdilution assay due to their relatively low MIC values in the previous experiments. To attain Atlantic salmon serum, 45 mL of previously extracted whole blood was subdivided into 15 mL tubes, incubated at room temperature for 30 minutes, and then placed at 4°C overnight. Coagulated blood was centrifuged at 900 xg for 10 minutes at 4°C. Serum was collected from above the cell pellet of each tube and pooled into a 50 mL tube. Serum was then heat inactivated using a previously described method [61].

The pathogen selected for this experiment was *E. coli*, due to its lowest reported MIC values and its nature as a model organism. Atlantic salmon serum was obtained from whole blood left to clot overnight at 4°C, centrifuged at 800 × g for 15 minutes at 4°C to remove any residual cells. The assay was carried out as described above with the substitution of Atlantic salmon serum for MHB.

3.2.6 Haemolysis assay

The haemolytic activities of synthetic peptides P9-4, P11-5, P11-6, and PG-1 were assessed as previously described [109, 139]. Briefly, 3 mL of heparinised whole blood was collected from visually healthy Atlantic salmon then centrifuged at 1,000 × g for 10 minutes at room temperature. The serum and buffy coat were removed and discarded. Red blood cells were washed in 10 mL of PBS and centrifuged at 1,000 × g for 10 minutes. This process was repeated three times, discarding the supernatant between washes. Erythrocytes were quantified using a haemocytometer and resuspended to 1×10^8 cells/mL in PBS or Atlantic salmon serum. Each peptide was serially diluted in sterile water to give final concentrations of 800, 400, 200, 100, 50, 25, 12.5 and 6.25 µM. 180 µL of the erythrocyte suspension and

20 µL of each concentration of the selected peptides was added to the desired wells of a V-shaped 96 well microtitre plate. Control wells contained 20 µL of 2% Triton-X 100 or PBS for 100% and 0% lysis, respectively. The plates were incubated for 2 hours at room temperature then centrifuged at $1,000 \times g$ for 10 minutes. 100 µL of the supernatant from each well was transferred to a fresh flat-bottom microtitre plate and the optical density was measured at 540 nm using a microplate reader. Percentage haemolysis was determined for each concentration of peptide by comparison to 100% and 0% lysis using the following formula: $[(A_{\text{synthetic peptide}} - A_{0\% \text{ lysis}}) / (A_{100\% \text{ lysis}} - A_{0\% \text{ lysis}})] \times 100$, where A is the absorbance at 540 nm.

3.2.7 *Peripheral blood leukocyte (PBL) assay*

The peripheral blood leukocyte (PBL) stimulation was assessed as previously described [65]. Briefly, 3 mL heparinised whole blood from visually healthy Atlantic salmon was collected and placed on ice. This sample was split into two 2 mL centrifuge tubes and progressively spun at $100 \times g$, $200 \times g$, and $400 \times g$ for 1 minute, then $800 \times g$ for 2 minutes. The buffy coat was harvested from each tube and pooled in fresh 1.5 mL centrifuge tubes, resuspended in 1 mL of RPMI, then centrifuged at $400 \times g$ for 5 minutes. The supernatant was removed and the final pellet was resuspended in 2 mL of RPMI and split into two 5 mL tubes. Cells were quantified with a hemocytometer then diluted with RPMI containing FBS (66% FBS/33% RPMI) to a final concentration of 1×10^7 PBLs/mL and 1×10^7 RBCs/mL to a total volume of 3 mL. Selected peptides (P9-4, P11-5, P11-6) were serially diluted in 1xPBS to give final concentrations of 0, 0.5, 5, 50, and 500 µM. Each peptide concentration was added to 100 µL of cells to each well of a flat bottom 96 well microtitre plate, in duplicate, and incubated at room temperature for 6 hours. No-stimulation controls were included for each time point, with 10 µL of PBS instead of peptide. After 6 hours, cells were titrated to

resuspend the monolayer and transferred to 1.5 mL centrifuge tubes. Total RNA was extracted with 400 µL nucleic acid extraction buffer (4 M urea, 1% SDS, 0.2 M NaCl, 1 mM sodium citrate pH 7.5) containing 20 U of proteinase K (Bioline) per sample. After digestion protein, cellular debris, and detergent were removed by centrifugation in 7.5 M ammonium acetate at $14,000 \times g$ for 5 minute, and nucleic acids were recovered by isopropanol precipitation at $16,000 \times g$ for 10 minute followed by an ethanol wash of the nucleic acid pellet. Complete removal of DNA was ensured by treatment with 4 units Baseline-Zero DNase (Epicentre) for 30 minutes at 37 °C before the DNase was removed and the RNA re-precipitated in 2.5 M LiCl and washed twice in 70% ethanol. The remaining ethanol was removed, pellets were resuspended in 12 µL distilled water with RNA secure, and incubated at 55°C for 5 minutes with occasional vortexing. Total RNA was quantified and visualized on a 1% agarose gel. First strand cDNA was synthesised from total RNA (10 µL) using BioScript reverse transcriptase (Bioline, NSW, Australia) with Oligo (dT)18 priming according to manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed on cDNA reverse-transcribed from total RNA. The relative expression of IL-8 was measured by qPCR using SYBR Green chemistry using CFX Connect™ Real-time PCR Detection System (Bio-Rad, NSW, Australia). Table 3 lists the primers for IL-8 detection and of the reference gene β -actin. qPCR reactions consisted of 20 µL volumes using a 2x SensiMixPlus SYBR & Fluorescein PCR master mix (Bioline), forward and reverse primers (200 nM of each) and 1 µL of cDNA. The amplification program was as follows: 95°C for 2m to activate the DNA polymerase followed by 40 cycles of 95°C for 5s, 60°C for 20s. At the end of the cycling protocol melt curve analysis was run to ensure amplification specificity. mRNA expression levels were normalised using the mean expressions of the reference gene, which maintained stable expression [140]. The qPCR data were analysed using R Studio (qPCR package) using

methods described previously [141, 142]. Data were graphed using GraphPad Prism 5 (GraphPad Software, Inc.).

Table 1. Oligonucleotide primers used in real-time PCR experiments.

Target mRNA	Accession #	Forward primer (5' → 3')	Reverse primer (5' → 3')
β-actin	AF012125	TTGCGGTATCCACGAGAC	TAGAGGGAGCCAGAGAGG
IL-8	BT046706	ACCAGCGAGATAACAA	CCAGGAGCACAATGACAA

3.3 Results

3.3.1 Antibacterial properties of synthetic peptides

All of the synthetic peptides exhibited low MIC values (100 µM or lower) in the absence of salt (Table 4). When the antimicrobial activities were estimated under saline conditions, P9-4 and PG-1 showed a notable increase in stability towards salts against *A. hydrophila* and *Y. ruckeri* with MICs of 40µM and 5-20µM respectively. All peptides showed little or no antimicrobial capacity in the presence of Atlantic salmon serum (Figure 8).

Table 4. Antibacterial activity of synthetic peptides (µM) in the absence/presence of NaCl.

Peptide	0 mM	300 mM	0 mM	300 mM	0 mM	300 mM	0 mM	300 mM
Pathogen	<i>A. hydrophila</i>	<i>A. hydrophila</i>	<i>E. coli</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	<i>Y. ruckeri</i>	<i>Y. ruckeri</i>
P9-4	10	40	2.5	5	5	40	10	40
P11-5	10	>80	2.5	40	10	>80	20	>80
P11-6	10	>80	2.5	20	5	>80	5	>80
HHC10	20	>80	2.5	5	10	>80	10	>80
K6L2W3	10	>80	2.5	20	10	>80	10	>80
K7LW3	80	>80	2.5	40	40	>80	2.5	40
PG-1	40	20	2.5	2.5	5	>80	5	5

Pep-1-K	40-80	>80	5	80	20	>80	5	40-80
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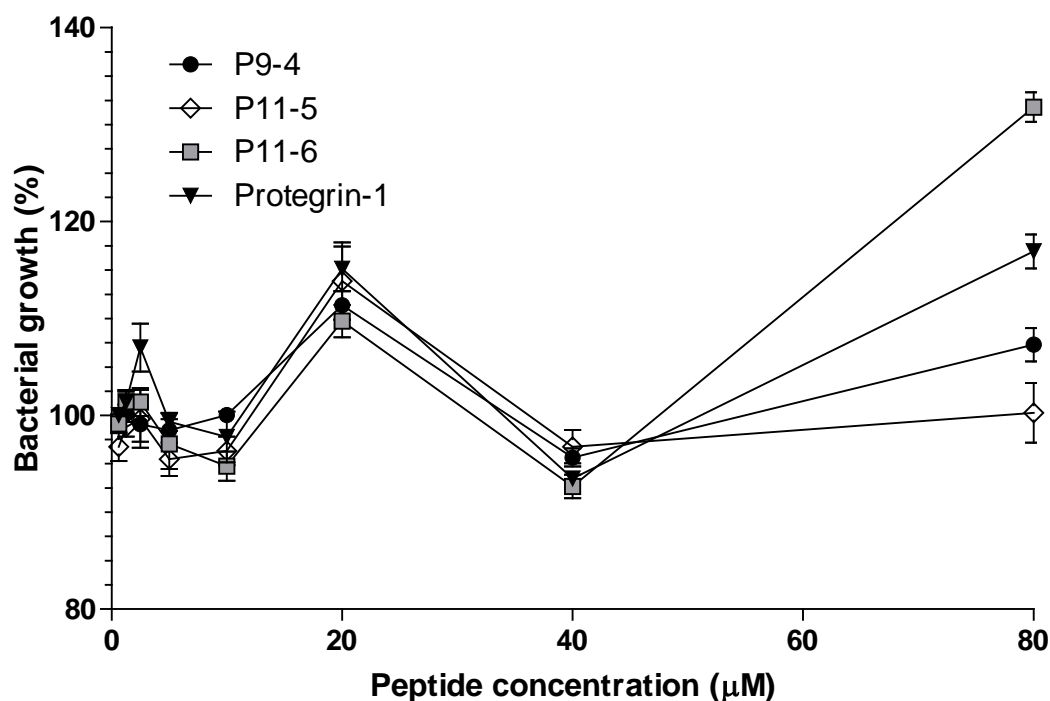


Figure 8. Antimicrobial activity of the peptides P9-4, P11-5, P11-6, and PG-1 against *E.coli* in the presence of Atlantic salmon serum. Data shown are the means \pm SD of duplicate wells.

3.3.2 Haemolytic activity of synthetic peptides

The synthetic peptides P9-4, P11-5, P11-6, and PG-1 were chosen as candidates for cytotoxicity testing due to their relatively low MIC values in previous experiments. To better understand how these peptides may interact with eukaryotic cells, we determined their ability to lyse Atlantic salmon erythrocytes in both RPMI and heat treated Atlantic salmon serum. All peptides were haemolytic to Atlantic salmon erythrocytes (Figure 9). P9-4 caused haemolysis of Atlantic salmon erythrocytes at concentrations as low as 2.5 μ M, where

haemolysis was 19.3%, and up to 42% haemolysis at higher concentrations. The lower cytotoxicity exhibited by P11-5 and P11-6 agreed with the previously reported values in human red blood cells [102], showing 22.7% and 20.2% haemolysis at concentrations as high as 80 μ M. PG-1 shows haemolysis as high as 40.2% at 80 μ M. In contrast the haemolytic activity of all peptides was absent in the presence of Atlantic salmon serum at all concentrations (data not shown) after 48h.

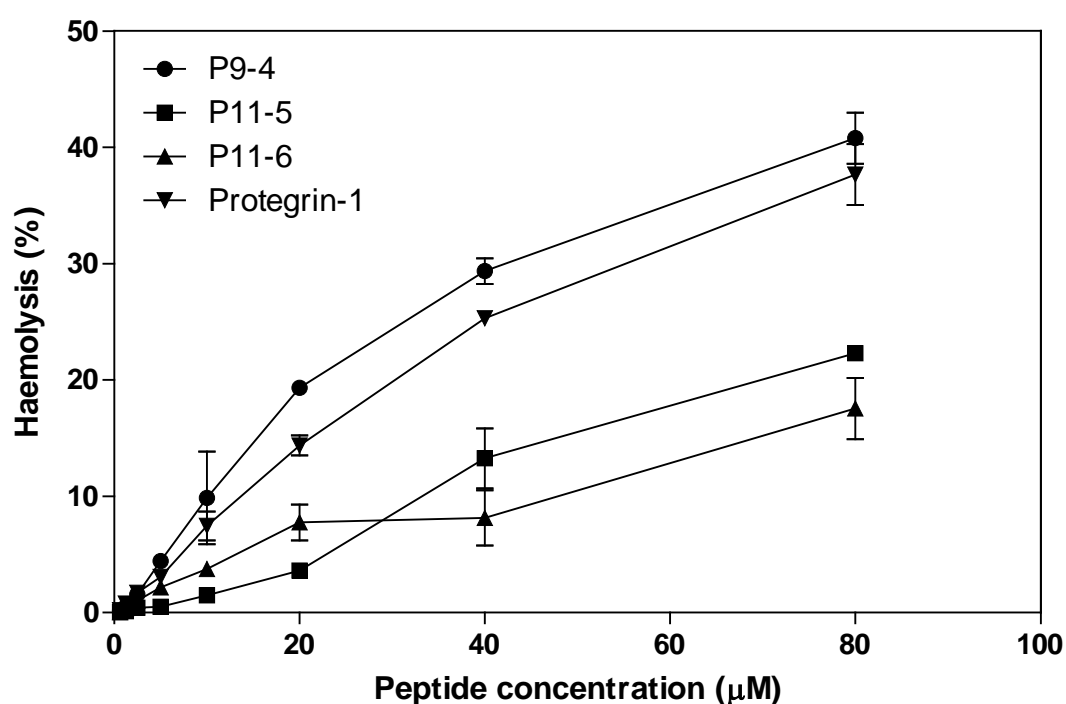


Figure 9. Haemolytic activity of the peptides P9-4, P11-5, P11-6, and PG-1 to Atlantic salmon erythrocytes in RPMI. Data shown are the means \pm SD of duplicate wells.

3.3.3 Host immunomodulatory activities of synthetic peptides

To investigate the possible influence of synthetic peptides on the interleukin-8 production, we stimulated a combination of PBLs and RBCs (1.3×10^7 PBLs/mL and 1×10^7 RBC/mL respectively) for 6 h with an array of concentrations of P9-4, P11-5, and P11-6 in

conventional media (RPMI) and in FBS supplemented RPMI. IL-8 expression was significantly upregulated at 6 h post-stimulation at 500 μ M with P9-4 and P11-5 in serum. P9-4 induced a 4 fold increase in IL-8 expression and P11-5 showed a 15 fold increase in IL-8 expression at the same concentration (Figure 10). P11-6 showed no biologically significant increase (2 fold or greater) in IL-8 expression in either RPMI or FBS with RPMI. All peptides tested showed no significant effect on IL-8 in conventional media.

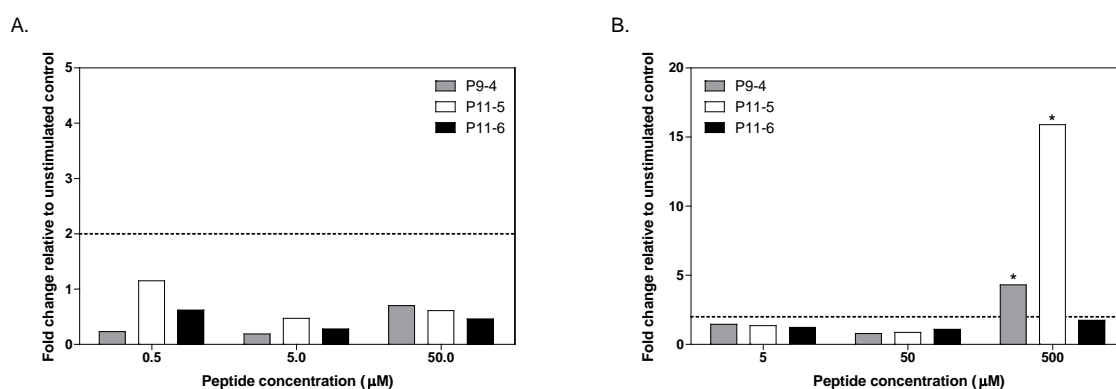


Figure 10. IL-8 gene expression in Atlantic salmon peripheral blood leukocytes stimulated with P9-4, P11-5, and P11-6. Gene expression was assessed by real-time PCR at 6 h post incubation with P9-4, P11-5, and P11-6 at 0.5 μ M, 5 μ M and 50 μ M in RPMI media(A) and 0.5 μ M, 5 μ M and 50 μ M in 66% FBS supplemented with 33% RPMI(B). Expression was normalised to the mean expression of β -actin reference gene. Data shown are the means \pm SE of quadruplicate PBL samples assayed in duplicate by quantitative real-time PCR and presented as fold induction compared to unstimulated control (PBS). * indicates a significant fold induction ($P < 0.05$) compared to unstimulated control while the dotted line represents the minimum fold change (2 fold) deemed biologically significant.

3.4 Discussion

In the present study, the antimicrobial activity of short synthetic cationic peptides P9-4, P11-5, P11-6, and PG-1 were effective in inhibiting two common pathogens of Atlantic salmon, *A. hydrophila* and *Y. ruckeri* at relatively low concentrations [34, 102, 105, 106,

143]. It was previously shown that each of these peptides possesses potent antibacterial activity *in vitro* against mammalian bacterial and fungal pathogens and improved stability in the presence of salts [102, 105]. Additionally, the results of this study are in general agreement with the antimicrobial activity of these peptides and the previously reported MIC values for the representative pathogens *E.coli* and *P. aeruginosa*. In comparison, the naturally derived Atlantic salmon cathelicidins (asCATH1 and asCATH2) showed no antibacterial activity to the same strain of *Y. ruckeri* up to 80 μ M [65]. Furthermore, these peptides retain their potent antimicrobial activity when subjected to saline conditions. This is notable because many AMPs, including other synthetic analogs, lose their potent antimicrobial activity when subjected to physiological salt conditions and are antagonized by mono or divalent cations [48]. Additionally, peptides which exert antimicrobial activity in artificial media have been shown to lose this activity or function differently within blood or serum [36, 61, 144]. Peptide inactivation or degradation may be due to proteolytic degradation or other inherent factors present in serum [44, 61]. While peptides exhibited low MICs against *E. coli* in serum-free media, their potent antimicrobial activity was diminished in the presence of serum. While PG-1 has been shown to overcome this inhibitory effect at concentrations of PG-1 at 100 μ g/mL [145], the findings of this work suggest that direct application of these peptides at epithelial surfaces may show the highest efficacy against bacterial pathogens.

Low toxicity to host cells is an important trait of any potential therapeutic agent. It has been shown that the cell specificity of AMPs is influenced by the net positive charge and hydrophobicity of peptides and a balance between the two was necessary to retain potent AMPs and avoid hemolytic activity [47]. While some of the peptides in this study caused

haemolysis of salmon erythrocytes in PBS, this activity was greatly diminished in the presence of serum. Importantly, the haemolytic activity of all peptides was absent in the presence of Atlantic salmon serum at all concentrations after 48 hours (data not shown). This suggests that haemolytic activity is unlikely to occur *in vivo* and that potential host cell degradation commonly associated with these peptides would be theoretically limited.

Despite showing diminished antimicrobial activity in the presence of serum, IL-8 expression was significantly upregulated at 6 hours post-stimulation at 500 μ M for P9-4 and P11-5 in conventional media supplemented with FBS. IL-8 expression has been previously reported to be regulated by naturally occurring AMPs (cathelicidins) in humans and Atlantic salmon. IL-8, one of the first CXC chemokines to be discovered in fish, is secreted by immune related cells upon interaction with a pathogen and is suggested to attract lymphocytes and monocytes to the source of infection whereas IL-8 in mammals usually attracts neutrophils [34]. To my knowledge, this is the first study to show induction of cytokine expression using short synthetic peptides in fish. In mammalian studies, IDR-1, a synthetic HDP, was also shown to enhance the levels of infection-clearing chemokines. While not directly antimicrobial *in vitro*, IDR-1 activated several signaling pathways, enhancing expression levels of chemokines and suppressing the levels of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α [146].

Similarly, previous work using human alpha-Defensin-1 (HNP1), another well-known HDP, demonstrated the ability of AMPs to influence immune-related genes and modulate the expression of genes encoding proinflammatory cytokines, chemokines, and IFN-stimulated genes in rainbow trout [111]. Similarly, defensins used as adjuvants in mice conferred resistance and acted as chemoattractants for resting human T cells both *in vivo*

and *in vitro* [147]. In mammals, chemokines have been widely used as adjuvants in vaccines against viral infections attracting immune-related cells to the site of inflammation and regulating the immune functions of the recruited cells [148]. Modulation of pathogen responsive genes and protein expressions may reduce the severity or enable the complete protection against pathogen infections. A better understanding of IL-8 expression in response to synthetic peptides might enable their use to regulate inflammatory cytokines and control localized inflammation during vaccination. While these data look promising, further examination of other inflammatory cytokines and immune related genes is necessary to better understand possible interactions within the host environment during stimulation with non-native peptides.

In summary, this study demonstrated the modulatory capabilities of two short synthetic cationic peptides (P9-4 and P11-5) in their ability to influence expression of the chemokine interleukin-8. Interestingly, the potent antimicrobial activity of P9-4, P11-5, P11-6, and PG-1 against *E. coli* is negligible in the presence of host serum. Furthermore, this study showed that while these peptides have some host haemolytic activity in conventional media, no haemolytic activity occurs in the presence of heat inactivated Atlantic salmon serum. The above considerations emphasize the validity and importance of assays which evaluate the antimicrobial activities of peptides under conditions potentially relevant to their development to function as therapeutic agents *in vivo*. Additionally, this study further demonstrated the efficacy of synthetic peptides as broad spectrum and immunomodulatory therapeutic agents with potentially useful aquaculture applications.

3.5 Acknowledgements

We thank Philip Crosbie and Melanie Leef for their assistance in procuring Atlantic salmon blood. We would also like to thank Mike Williams for assistance in acquiring the bacterial strains used in this study.

Chapter 4: Short synthetic cationic peptides increase phagocytosis, cell proliferation and respiratory burst in Atlantic salmon (*Salmo salar* L.) head kidney leukocytes

4.1 Introduction

While the majority of past research focused on the direct antimicrobial aspects of AMPs, recent research has targeted the immunomodulatory role of bioactive peptides during an innate immune response. In particular, defensins and cathelicidins have demonstrated their ability to not only induce a proinflammatory immune response in a wide variety of immune cells via the production of cytokines but also to suppress the release of proinflammatory mediators induced by LPS and other bacterial products. Additionally, they have been shown to promote cell proliferation and phagocytosis by immune-competent cells and assist in their ability to differentiate between host and foreign nucleic acids [52, 57-59, 64]. Because of the multifaceted mechanisms of these molecules, it has been suggested that peptides which exhibit primarily antimicrobial activity be referred to as AMPs whereas the term host defense peptides (HPDs) should be used for those peptides with immunomodulatory abilities [95].

Although these natural peptides exhibit potential as anti-infective agents, the commercial application of these peptides has been limited for a variety of reasons. While many AMPs have been shown to inhibit the growth of bacteria at relatively low minimum inhibitory concentrations (MIC) *in vitro*, they fail to confer the same level of protection *in vivo* [24, 94]. Moreover, their amino acid composition and non-selective nature has shown them to be haemolytic to host cells at higher concentrations, and are susceptible to inactivation via salts at physiological concentrations and other components found naturally in blood and serum [36, 44, 45, 65]. Arguably, the single largest issue related to natural peptide production is the high cost associated with their synthesis. Peptides are commonly produced through solid-phase chemical synthesis and to a lesser extent recombinant DNA

methods using biological production systems [24, 95, 96]. Further, many require post-transcriptional modification to produce functional mature peptides [96]. In short, the purity and scale of production limit the use of naturally occurring AMPs as therapeutics.

The development of non-natural or synthetic AMPs is emerging as an effective strategy to circumvent many of the issues associated with naturally-produced AMPs. The design and production of synthetically derived peptides has been extensively reviewed in the literature [43, 95, 103, 104, 106]. These peptides can be optimized through amino acid substitution to produce small, cost effective peptides with increased antimicrobial activity [149, 150] and target specificity [103, 145], low cytotoxicity [102], notable salt tolerances [143, 149, 151], improved bioavailability [44, 151], and immunomodulatory capabilities [152-154]. While the use of synthetic peptides has been documented in mammalian models, there are currently no studies which test the capabilities of these peptides in teleost fish.

The aim of this study was to assess the ability of a range of small synthetic cationic peptides to modulate the immune functions of head kidney leukocytes in Atlantic salmon (*Salmo salar*). Functional assays to assess the direct stimulatory capacity of these peptides on immune competent cells were undertaken to explore their potential use as immunostimulatory compounds in fish.

4.2 Materials and Methods

4.2.1 Ethics statement

All work involving animals was approved by the University of Tasmania Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

4.2.2 Peptides, Peptide Synthesis, and Reagents

Peptides were synthesized by Life Research Australia (VIC, Australia) using solid-phase standard 9-fluorenyl methoxy carbonyl chemistry and purified to >95% by reverse-phase high-pressure liquid chromatography (Table 5). Mass spectral analysis and HPLC chromatography were used to ensure purity and the identity of each peptide.

Oligodeoxynucleotide 1668 (TCCATGACGTTCTGATGCT), phosphorothioated to increase resistance to nuclease degradation, was purchased from Sigma Genosys (Castle Hill, NSW, Australia). It was selected as a positive control based on its proven stimulatory abilities in Atlantic salmon, rainbow trout, other teleost fish, and murine studies [118-121].

Table 5. List of synthetic AMPs used in this study.

Peptide	Sequence	Molecular weight (g/mol)	Purity (%)	Notes	Ref.
P9-4	Ac-KWRRWIRWL-NH ₂	1440.78	95.50	improved stability in the presence of salts low cytotoxicity compared to reference peptide	[102]
P11-5	Ac-GKLFKKILKIL-NH ₂	1341.80	96.89		[102]
P11-6	Ac-KKLIKILKIL-NH ₂	1378.91	96.97		[102]
HHC10	Ac-KRWWKWIRW-NH ₂	1485.82	99.27	protects mice against invasive <i>S. aureus</i> infections through intravenous (IV) injection	[106]
K ₆ L ₂ W ₃	Ac-KLWKKWKKWLK-NH ₂	1613.09	96.84	suppressed LPS-induced inducible nitric oxide synthase (iNOS) mRNA expression and inhibited release of nitric oxide (NO) following LPS stimulation in RAW264.7 cells	[103]
PG-1	Ac-RGGRLCYCRRRFCVC VGR-NH ₂	2201.68	96.54	synergistically enhanced respiratory burst when coupled with endotoxin in human monocytic cell lines	[50]

4.2.3 *Fish*

Atlantic salmon weighing approximately 200-300 g were maintained in a 3000 L freshwater water re-circulating tank with biofilter at IMAS Launceston, University of Tasmania, Launceston, Australia.

4.2.4 *Isolation of head kidney leukocytes*

Head kidney leukocytes were isolated as previously described [122]. Briefly, head kidney was aseptically removed from each fish and passed through a 100 μ M sterilized metal mesh repeatedly using L-15 medium containing 1% penicillin and streptomycin (P/S) (Sigma), 0.2% heparin (20 U/mL) (Sigma). Cell suspensions were then placed on a 34/54% Percoll (Sigma) gradient and centrifuged at 400 g for 40 minutes at 4°C. After centrifugation, cells were harvested from the 34/54% interface and washed twice in phosphate buffered solution (PBS, Sigma). Cells were quantified by trypan blue exclusion using a haemocytometer and resuspended in the L-15 media mentioned above for use in their respective assays.

4.2.5 *Phagocytosis assay*

Phagocytosis was assessed using a modified previously described method [123]. Briefly, cells were seeded in 96-well plates at a density of 1×10^7 cells per well, and allowed to adhere at 18°C for 3 hour. Wells were then washed three times with L-15 medium containing 1% penicillin and streptomycin to remove any non-adherent cells. Cells were stimulated with a synthetic peptide (5 μ M) or CpG-ODN 1668 (5 μ M) for 4 hour at 18°C. Control leukocytes were incubated with L-15 medium alone. After the incubation period, 20 μ L Congo red-stained yeast cells (10^7 cells/mL) were added to each well. To monitor

phagocytosis, two pictures were taken randomly by a Cannon 600D Digital SLR with an optical adapter equipped to a light microscope at given time points. The phagocytic activity (PA) was determined by the percentage of cells with engulfed yeast cells (PA = number of phagocytic cells with engulfed bacteria/number of phagocytes). The phagocytic index (PI) was determined by the number of engulfed yeast cells per cell (PI = number of engulfed yeast/phagocytic cells).

4.2.6 *Respiratory burst activity assay*

Respiratory burst activity was assessed by nitro blue tetrazolium (NBT) (Sigma) reduction to formazan after stimulation of cells as previously described [123]. Briefly, cells were isolated and 96-well plates were prepared and stimulated for 4 hours at 18°C as stated above. Untreated leukocytes were used as the negative control and leukocytes treated with phorbol myristate acetate (PMA) (100 ng/mL) as the positive control. After stimulation, the cell monolayer was washed twice with PBS to remove any residual stimulant and 100 µL of NBT solution (1 mg/mL in L-15) was added per well. Cultures were then incubated at 18°C for 1 hour. After incubation, cells were fixed with methanol after the removal of the NBT solution. Cells were then air dried for 20 minutes then washed again to remove residual methanol. Finally, 120 µL 2M KOH and 140 µL of DMSO (Sigma) were added to each well and the optical density was measured with an Infinite M200 Pro microplate reader (Tecan) at 620 nm.

4.2.7 *Cell proliferation assay*

Cell proliferation was assessed by measuring total nucleic acid content of lysed cells after stimulation treatments using a fluorometer. Briefly, cells were seeded in 96-well plates

at a density of 1×10^5 cells per well, and allowed to adhere at 18°C for 3 hours. Wells were then washed three times with L-15 medium containing 1% penicillin and streptomycin and to remove any non-adherent cells. Cells were stimulated with a synthetic peptide (5 μ M) or CpG-ODN 1668 (5 μ M) for 5 days at 18°C. Control leukocytes were incubated with L-15 medium alone. Cells were immediately frozen after stimulation. Total nucleic acid was extracted as previously described with modification [124]. Briefly, previously stimulated 96-well plates were thawed and 300 μ L nucleic acid extraction buffer (4M urea, 1% SDS, 0.2 M NaCl, 1 mM sodium citrate pH 7.5) containing 20 U of proteinase K (Bioline) per sample was added to each well. 96-well plates were then placed on ice and allowed to digest for 30 minutes. Samples were transferred from their wells into 1.5 mL microcentrifuge tubes. Protein, cellular debris, and detergent were removed by centrifugation in 7.5M ammonium acetate at 14,000 x g for 5 minutes, and nucleic acids were recovered by isopropanol precipitation at 16,000 x g for 10 min followed by an ethanol wash of the nucleic acid pellet. Complete removal of RNA was ensured by treatment with 4 units RNase A (Sigma) for 30 min at 37°C. Samples were then resuspended in molecular water and quantified using a Qubit fluorometer (Invitrogen) using the manufacturer's protocol.

4.2.8 Statistical analysis

Statistical analysis was carried out using GRAPHPAD™ PRISM version 5.00 for WINDOWS® (GraphPad Software). Differences between groups were determined using a one-way ANOVA with a Tukey posthoc test. Data were subject to a Shapiro–Wilk's test for normality and a Levene's test for equality of variances to satisfy the assumptions required for an ANOVA. Results were considered significant if $P < 0.05$.

4.3 Results

4.3.1 Phagocytosis assay

Phagocytic activity of head kidney leukocytes stimulated with various synthetic cationic peptides (5 μ M), CpG-ODN 1668 (5 μ M) or an untreated cell control differed significantly between treatments ($F_{[7, 16]} = 18.79$, $P < 0.0001$; Figure 11A). A Tukey's post-hoc analysis of the groups indicated that there was a significant increase in the phagocytic activity of the groups stimulated with K₆L₂W₃ and HHC-10 compared to the untreated control group and all other peptides tested ($P < 0.05$). In addition CpG-ODN 1668 had a significantly higher phagocytic activity than all other treatments ($P < 0.05$). Moreover, differences in phagocytic index existed between treatments ($F_{[3, 8]} = 0.6703$, $P = 0.5938$; Figure 11B) with the groups stimulated with K₆L₂W₃ and HHC-10 showing significantly improved phagocytic index in comparison to the untreated group as well as PG-1, P11-5, and CpG-ODN 1668.

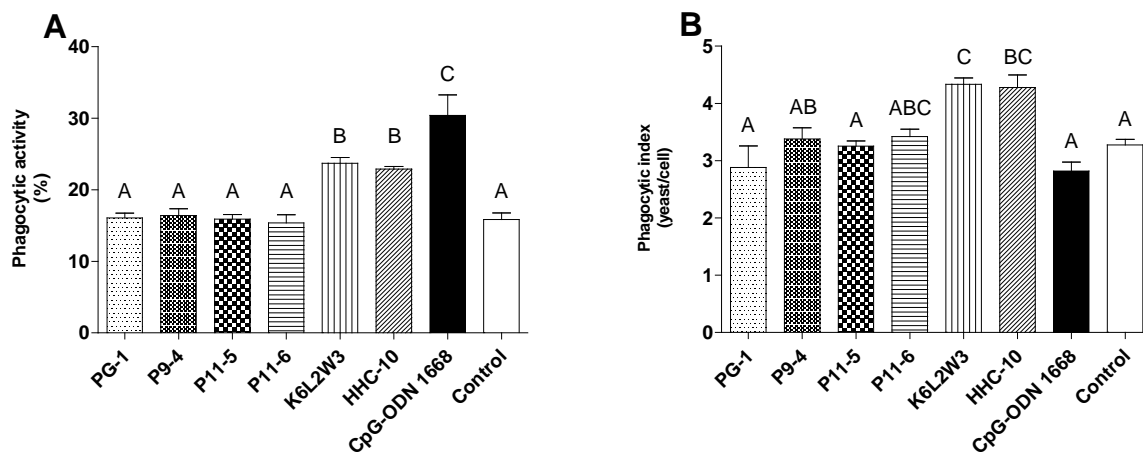


Figure 11. Phagocytic activity (A) and phagocytic index (B) of Atlantic salmon (*S. salar*) head kidney leukocytes stimulated with individual synthetic AMPs (5 μ M), or CpG-ODN 1668 (5 μ M). The phagocytic activity (PA) was determined as the percentage of cells with engulfed yeast cells. The phagocytic index (PI) was determined as the number of engulfed yeast cells per cell. Control leukocytes were incubated with medium alone. Data shown are the means

\pm SE of quadruplicate HKL samples assayed from 3 fish. Different superscript letters indicate significant differences between the groups ($P < 0.05$).

4.3.2 Respiratory burst assay

Respiratory burst activity of head kidney leukocytes stimulated with stimulated with various synthetic cationic peptides (5 μ M), CpG-ODN 1668 (5 μ M), PMA (100 ng/mL), or an untreated cell control differed significantly between treatments ($F_{[7, 16]} = 56.07$, $P < 0.0001$; Figure 12). A Tukey's post-hoc analysis indicated that there was a significant increase in the NBT reduction by the groups stimulated with PG-1, P11-6, K₆L₂W₃, and HHC-10 compared to the control but not to the groups stimulated by P9-4 and P11-5 ($P < 0.05$). The positive control, PMA, was significantly different than all other treatments ($P < 0.05$). There was no significant difference between P9-4, P11-5, and the untreated control.

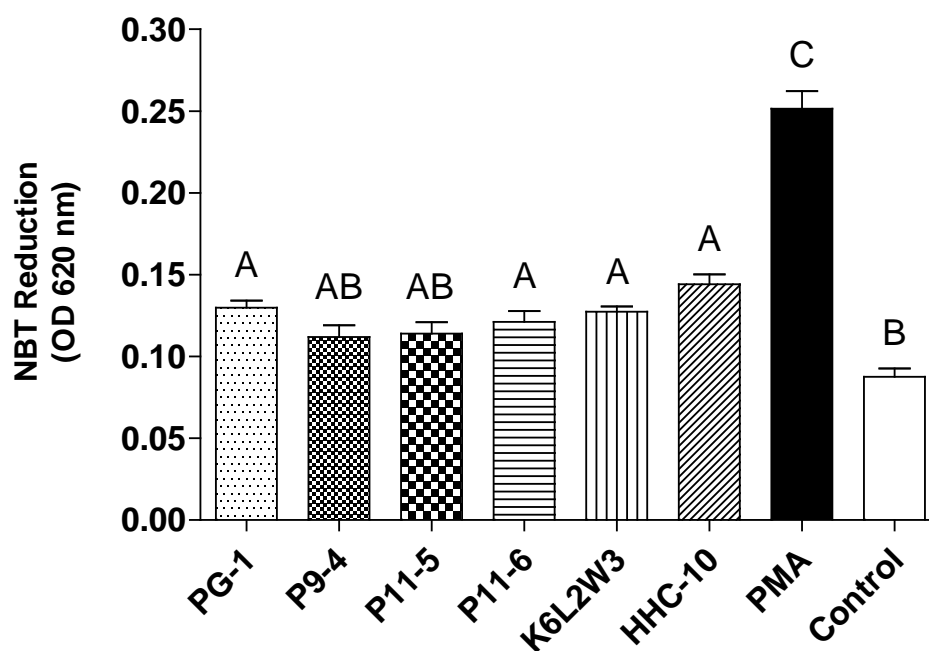


Figure 12. Respiratory burst activity of Atlantic salmon (*S. salar*) head kidney leukocytes stimulated with individual synthetic AMPs (5 μ M), or CpG-ODN 1668 (5 μ M). Control leukocytes were incubated with medium alone and positive controls were treated with PMA (100 ng/ml). Data shown are the means \pm SE of quadruplicate HKL samples assayed from 3

fish at 620 nm after 60 minutes incubation. Different superscript letters indicate significant differences between the groups ($P < 0.05$).

4.3.3 Cell proliferation assay

Proliferation of head kidney leukocytes stimulated with various synthetic cationic peptides (5 μ M), CpG-ODN 1668 (5 μ M), or an untreated cell control differed significantly between treatments ($F_{[7, 16]} = 36.99$, $P < 0.0001$; Figure 13). A Tukey's post-hoc analysis of the groups indicated that there was a significant increase in total DNA yield from cells treated with HHC-10 compared to the peptides P9-4, P11-6, and the untreated control but not PG-1, P11-5, and K₆L₂W₃ ($P < 0.05$). Cells stimulated with CpG-ODN 1668 had a significantly higher total DNA yield than all other treatments ($P < 0.05$).

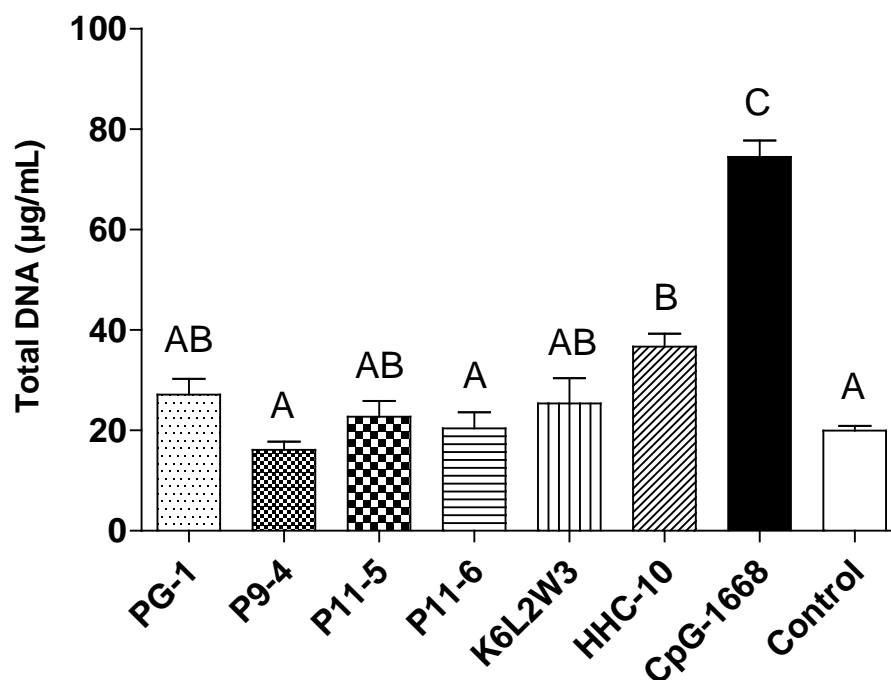


Figure 13. Cell proliferation of Atlantic salmon (*S. salar*) head kidney leukocytes stimulated with individual synthetic AMPs (5 μ M), or CpG-ODN 1668 (5 μ M) for 5 days. Control leukocytes were incubated with medium alone. Data shown are the means \pm SE of quadruplicate HKL samples assayed from 3 fish. Different superscript letters indicate significant differences between the groups ($P < 0.05$).

4.4 Discussion

Phagocytic cells play an integral role in the destruction, clearance, and mediation of bacterial pathogens. During an infection, phagocytes are triggered by cytokines or bacterial components to migrate to inflammatory sites. Once there, phagocytes attach to foreign particles and ingest them. Intracellular destruction of pathogens is achieved by a variety of bactericidal mechanisms including the production of AMPs, degradative enzymes, oxygen intermediates, or nitrogen intermediates [10, 155].

In the current study, the ability of novel synthetic cationic peptides to stimulate the phagocytic process of Atlantic salmon head kidney leukocytes *in vitro* was demonstrated for the first time. Peptides K₆L₂W₃ and HHC-10 significantly increased phagocytic activity of adherent head kidney leukocytes as compared to the other peptide treatments and the untreated controls. Additionally, cells stimulated with these peptides ingested more yeast per phagocytic cell with some variability between peptide treatments. HHC-10 increased cell proliferation in leukocytes stimulated for 5 days. Moreover four of the six peptides tested were capable of significantly influencing respiratory burst activity. The production of superoxide anion and the conversion of this into a large variety of reactive oxygen species (ROS) by phagocytes is a result of the activation of the NADPH oxidase complex and is generally associated with immune stimulation [10, 37]. This may represent a non-specific response by these cells to foreign peptides, as some but not all of these peptides elicited a response in other phagocytic functions.

Due to the novel nature of these peptides and their recent emergence, only a handful of scientific papers discussed the use of these specific peptides with most of their

putative functions closely associated to mammalian biology and drug development [102, 103, 156]. Many of the methods used in mammalian studies lie well beyond the scope of current fish immunological studies. Nonetheless, some interesting findings and implications relevant to teleosts are still apparent. HHC-10, a *de novo* designed peptide, demonstrated significant *in vitro* inhibitory activity against antibiotic-resistant “Superbug” bacteria (i.e. strains of exceptionally resilient bacteria) in preliminary work and in many cases outperformed many of the most potent conventional antibiotics commonly used to treat these bacteria [106]. Furthermore, HHC-10 demonstrated substantially lower toxicity compared to MX-226, a peptide from advanced clinical trials, and conferred significant protection when administered to mice through intravenous (IV) injection [106]. In the only other available study involving this peptide, HHC-10 was capable of inhibiting the *M. tuberculosis* complex bacteria *in vitro* and *in vivo* [156]. Additionally, it was shown that HHC-10 administration to infected mice resulted in a significant reduction of IFN- γ transcription [156]. Previous research involving K₆L₂W₃, an 11-mer cationic Trp-rich peptide modelled after Indolicidin, suggested this peptide might have anti-inflammatory activities. While our results showed that this peptide significantly induced respiratory burst through the production of superoxide anion in salmon leukocytes, K₆L₂W₃ suppressed LPS-induced nitric oxide synthase (iNOS) mRNA expression and inhibited release of nitric oxide (NO) following LPS stimulation in RAW264.7 cells [103]. Additionally, PG-1 was previously shown to neutralize TNF- α or nitric oxide, in a dose-dependent manner, when induced by endotoxin. However, macrophages co-treated with combinations of PG-1 and endotoxin had enhanced the respiratory burst and increased ROS release [50]. These results indicate that priming of immune cells by synthetic peptides may result in varying and multifaceted effects on immune function including the induction and suppression of both pro- and anti-

inflammatory responses according to different cellular responses [97]. Moreover, while our method provides a simple and straight forward measurement of superoxide anion, no measurement of nitric oxide or other ROS was conducted. Future work should target specific cellular receptors which may indicate the induction pathway stimulated by these peptides in fish. Furthermore, the application of more advanced cell recognition technologies such as flow cytometry may provide a better understanding of potential cell specific activation of synthetic cationic peptides. This information could lead to the development of peptides with more selective, cell specific stimulation and therefore a more targeted approach for applications such as immunostimulants or adjuvants.

It is important to note that while only a few researchers used the same specific peptides as this study, there is increasing evidence for the immunomodulatory properties of synthetic cationic peptides, such as the innate defense regulators (IDRs). Three key examples of IDRs which show immunomodulatory properties are IDR-HH2, IDR-1002, and IDR-1018. IDR-HH2, IDR-1002, and IDR-1018 promoted the release of other host defense peptides (HDPs) such as human α -defensins, and LL-37 as well as enhanced neutrophil-triggered killing of *E. coli in vitro* [157]. Additionally, IDR-1018 induced macrophage differentiation exhibited a unique combination of immune responses including production of both pro and anti-inflammatory mediators and increased phagocytosis of apoptotic cells in human macrophages [158]. Moreover, these peptides suppressed LPS-induced proinflammatory cytokine and ROS production in neutrophils whereas the control peptide IDR-1035 did not. These findings provide further evidence for peptide specific activation of immune cells as well as exhibit the regulatory capabilities of these peptides.

4.5 Conclusion

In conclusion, the capability of synthetic cationic peptides to improve the immune functions of Atlantic salmon leukocytes *in vitro* was demonstrated. Our results indicate that K₆L₂W₃ and HHC-10 significantly enhanced leukocyte phagocytic activity and phagocytic index. Additionally, PG-1, P11-6, K₆L₂W₃ and HHC-10 improved respiratory burst through increased superoxide anion production while HHC-10 alone increased cell proliferation in *in vitro* tests. While these findings provide preliminary evidence for the use of short, cationic synthetic AMPs as inducers of improved phagocytic function in fish, further experimentation is required to elucidate the mechanism of their activity and better understand the non-specific effects associated with these peptides.

Chapter 5: General Discussion

This work demonstrates that novel synthetic peptides and natural (synthesized) cathelicidins stimulate the phagocytic process of non-mammalian leukocytes *in vitro*. This study showed that selected synthetic AMPs and asCATH2 were capable of improving Atlantic salmon leukocyte phagocytic activity, increasing respiratory burst, and inducing cell proliferation *in vitro*. Furthermore, two synthetic peptides (P9-4 and P11-5) had the capacity to influence expression of the chemokine interleukin-8 in peripheral blood leukocytes (PBLs) *in vitro*. As all of these mechanisms play an important role in innate immunity. This work demonstrates the dynamic nature of these peptides to act not only as bactericidal agents but also potent effector molecules.

AsCATH2 was previously shown to increase expression of IL-8 in PBLs at biologically relevant concentrations *in vitro* [65]. The current study shows that this stimulatory ability extends to other immune cell lineages as well. Additionally, the functional differences highlighted by in this work positively reinforce the idea that asCATH2 primarily serves to modulate cell function whereas asCATH1 is directly antibacterial. While four of the six synthetic peptides tested were capable of significantly influencing respiratory burst activity, the notable standout from the functional assays was HHC-10 which also significantly improved phagocytic activity and increased cell proliferation after 5 days of stimulation. As mentioned previously (Chapter 4), HHC-10 was loosely developed based on a naturally occurring bovine peptide called Bac2A and variable amino acid substitution. This initial work produced two subsets of peptides, through SPOT synthesis, which contained nearly 1500 peptides [106]. The developers then utilized the amino acid sequences of these peptides to create quantitative structure-activity relationships (QSAR) models which relate biological relevant chemical characteristics to antimicrobial activity. Additionally, an artificial neural network was used to relate chemical descriptors to antimicrobial activity for the 100,000 computer generated peptides [106]. The result: a peptide with significant *in vitro* inhibitory activity against a variety of antibiotic-resistant bacteria and high *in vivo* efficacy when administered to mice (see Chapter 4). In the current study, HHC-10 significantly effected phagocytic cells and induced cell proliferation. In retrospect, this

peptide should have been considered for gene expression analysis whereas P9-4, P11-5, P11-6, and PG-1 were selected instead.

While this work demonstrates the ability of AMPs to directly stimulate and modulate phagocytic cell function, the mechanism by which this occurs is still unclear. Most likely AMPs act through the non-specific activation of PRRs. It is known that PRRs are able to trigger various innate immune responses including complement pathways, apoptosis, leukocyte activation and migration, and cytokine production [159]. These peptides, like many other soluble immune products, may have divergent effects on immune cells that depend on their concentration and the combinatorial effects elicited by the array of factors present at a particular site. Reactive oxygen and nitrogen intermediates act as potent signaling molecules at low doses and their role as inflammatory mediators to promote cell proliferation and differentiation is already evident in teleost fish [160]. While the exact mechanism and subsequent activation pathway is unknown in fish, in mammals this is largely accomplished through the modulation of expression of survival proteins through transcriptional regulators like NF- κ B and activation of signaling kinases that include MAP kinase and protein kinase C [161]. These kinases are integral to the regulation of antimicrobial functions that include cellular motility, phagocytosis, and cytokine production [161].

The second major goal of this thesis was to explore the use of short, synthetic cationic peptides as novel antibiotics for future aquaculture application (Chapter 3 and 4). Previous work involving the salmonid cathelicidins had already established their antibacterial capabilities against aquaculturally important bacterial pathogens [65]. Here it is shown that P9-4, P11-5, P11-6, and PG-1 were effective in inhibiting *A. hydrophila*, *Y. ruckeri*, *E.coli*, and *P. aeruginosa* at relatively low concentrations. Additionally, the antimicrobial activity seen against the latter two pathogens agreed with previously published data [34, 102, 105, 106, 143]. Despite the promising previous results, the potent antimicrobial activity of P9-4, P11-5, P11-6, and PG-1 was negligible in the presence of host serum. However, previous work involving PG-1 showed that this peptide protected mice from a

lethal challenge of *S. aureus* or vancomycin-resistant *Enterococcus faecium* (VREF) *in vivo*. In those studies, PG-1 was as effective as the conventional antibiotic vancomycin in treating MRSA-induced bloodstream infections and could be delivered up to 60 min after infection by intraperitoneal injection. It was demonstrated that the peptide did not translocate from the site of injection. This indicates that while the synthetic peptides seemingly underperformed in serum *in vitro*, they could be used for localized applications *in vivo*.

Many of the issues associated with peptide inactivation or loss of activity can be overcome during synthesis through modification. One method involves the incorporation of unnatural amino acids to improve protease stability. The addition of unnatural amino acids to peptide sequences greatly reduces or halts the proteolytic degradation process because they are not readily recognized by the binding sites of proteases [99]. Among the various unnatural α -amino acids, substitution of AMPs with D-amino acids is the most popular approach due to the commercial availability of the D-form of all 20 naturally occurring amino acids [99]. Additionally, the production of peptoids is another way to confer protease stability. Peptoids, or poly-N-substituted glycines, contain side chains appended to the nitrogen atom of the peptide backbone instead of the α -carbons in amino acids [99, 151, 162]. Notably, $K_6L_2W_3$ a peptide used in this thesis, has had both forms of modification done to it with interesting results. $K_6L_2W_3$ -D showed improved resistance to trypsin degradation compared to the parent peptide $K_6L_2W_3$ [103]. Furthermore, it along with $K_6L_2W_3$ inhibited LPS-induced nitric oxide synthase (iNOS) mRNA expression and the release of nitric oxide (NO) following LPS stimulation by binding to LPS in RAW264.7 cells [103]. The peptoid $k_6l_2w_3$ improved upon the design of its parent peptide by retaining potent antimicrobial activities against *E. coli* and *S. aureus* after trypsin treatment [162]. Subsequent studies investigating the direct antimicrobial activity of the peptides tested in this thesis might opt to redesign or modify the current peptides with either of the previously described methods to improve their resistance against degradation and thus potentially improve their application for biologically relevant conditions. Future work on the synthetic AMPs in Atlantic salmon could aim to develop signaling inhibitors in order to elucidate

cellular targets for peptide activation (both extracellular and intracellular). Additional research could explore the potential of aquaculturally-relevant bacteria strains to develop resistance towards these synthetic AMPs after multiple generations of subculture and treatment. This work would provide valuable information regarding the susceptibility of particularly problematic microbes towards these peptides.

While the likelihood that the AMPs in this work will be used as direct antibacterial agents beyond topical applications is low, due to their loss of bactericidal function in serum, these results indicate that their immunomodulatory capabilities demonstrate potential as adjuvants for vaccination. Adjuvants, substances which enhance host immune response to antigen, are used to improve vaccine efficacy by boosting the less-immunogenic potential of vaccines, extending the duration of protection, and/or directing the immune response [40, 163]. Administration of vaccines can be performed orally through feed incorporation, by immersion in diluted vaccine suspensions, or by intraperitoneal or intramuscular injection routes [164]. Oil emulsions such as Freund's complete or incomplete adjuvant (FCA/FIA) which contain mineral oil and a surfactant are commonly used in experimental injection vaccinations to generate long-term immune responses with various antigens [163, 164]. While cost effective, these adjuvants have been associated with a variety of severe side effects including injection site granuloma and localized toxicity in some species [165-167].

AMPs have been shown to both induce and inhibit a wide variety of inflammatory responses [147, 148]. While this work presents no direct evidence for the *in vivo* efficacy of the selected peptides used in this study, AMPs have already been targeted as potential novel adjuvant which could modulate the inflammation associated with vaccination and therefore limit localized damage commonly associated with conventional adjuvants [163]. A recent study showed that tilapia alpha-helical AMPs Oreochromicins are able to boost immune response in mammals and fish when co-administered with ovalbumin in mice and my32-Ls, a sea lice (*Lepeophtheirus salmonis*) antigen in fish [168]. Another study in zebrafish (*Danio rerio*) demonstrated that zfBD2 (zebrafish beta-defensin

2) possessed diverse immunomodulatory activity both *in vitro* and *in vivo* [92]. This beta-defensin in combination with a DNA vaccine conferred protection levels > 90% against a lethal spring viraemia of carp virus (SVCV) challenge. Moreover, while this DNA vaccine was previously shown to partially protect fish (50-80%) [169], co-administration with zfDB2 lowered the required dose of the vaccine from 10µg to 1µg and improved protection against SVCV [92]. Additionally, AMPs can be formulated with other immunogenic compounds/delivery methods to further improve their efficacy. Pleurocidin (PLE) and an outer membrane protein, glyceraldehyde-3-phosphate dehydrogenase produced recombinantly in *V. harveyi* (rGAPDH) were simultaneously encapsulated into microparticles using a biodegradable polymer and used to immunize grouper (*Epinephelus coioides*) against *V. harveyi*. Microparticles containing PLE conferred a significantly higher survival rate (85%) than that induced by microparticles containing rGAPDH alone (67%) [170]. In mammals, synthetic cell penetrating AMPs are being used as cationic delivery systems for immunogenic compounds including CpG-ODNs [171, 172] and TLR-agonists [173] with promising results.

With the rapid increase of technology, particularly in the areas of computer-aided peptide design, it is inevitable that many new potential peptides will emerge with improved functionality and cell specificity. Therefore, this thesis provides groundwork for those interested in this field with an emphasis on the functional applications of these peptides *in vitro*.

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